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PROCEEDINGS
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*FREQUENCY OF SEPARATION AND OF INTERLOCKING OF CLUSTERS OF GALAXIES**

By Jerzy Neyman and Elizabeth L. Scott

Statistical Laboratory, University of California, Berkeley

Communicated by S. Bochner, June 1, 1953

1. Introduction.—The present paper is a continuation of the study[1] by the present authors, to be referred to as Paper I, in which a probabilistic theory of the spatial distribution of galaxies was developed. Prior to explaining the postulates of this theory we mention a probabilistic concept that is used throughout the present paper. This is the concept of the probability generating function. For any non-negative integer-valued random variable μ the probability generating function is defined for $|t| \leqq 1$ as the expectation of t^μ and denoted by $G_\mu(t)$. The conditional probability generating function subject to a hypothesis H is denoted by $G_\mu(t|H)$.

The postulates of the theory are as follows.

Postulate (i): Galaxies occur only in clusters.

Postulate (ii): To every Borel measurable set R in space there corresponds a random variable $\gamma(R)$ that represents the number of cluster centers falling within R. The distribution of $\gamma(R)$ depends only on the measure, say $V(R) = V$ of R but not on any other properties of this set. If $R_1, R_2, \ldots$ is an at most denumerable sequence of disjoint Borel measurable sets, then the corresponding random variables $\gamma(R_1), \gamma(R_2), \ldots$ are completely independent.

The probability generating function of $\gamma(R)$ is denoted by $G_\gamma(t|V)$. It was found that, in order to satisfy *(ii)*, it is necessary and sufficient that for every set R of finite measure V

$$G_\gamma(t|V) = e^{Vh(t)} \tag{1}$$

with

$$h(t) = -h_0 + \sum_{k=1}^{\infty} h_k t^k \tag{2}$$

where $h_0 > 0$ and $h_k \geqq 0$, for $k = 1, 2, \ldots$ and

$$\sum_{k=1}^{\infty} h_k = h_0. \tag{3}$$

Postulate (iii): Let R_1 be a set of positive measure and R_2 a measurable subset of R_1. Let, further, $a_1, a_2, \ldots, a_m$ be an arbitrary combination of m numbers selected out of $1, 2, \ldots, n \geqq m$. Consider the case where R_1 is known to contain exactly n cluster centers, say $C_1, C_2, \ldots, C_n$. It is postulated that the conditional probability that R_1 will contain the m cluster centers $C_{a_1}, C_{a_2}, \ldots, C_{a_m}$, and no others, is independent of the particular combination $a_1, a_2, \ldots, a_m$ but depends only on n, m and, naturally, on the measures of R_1 and R_2.

Postulate (iv): The number ν of galaxies belonging to a given cluster is a random variable capable of assuming any positive integer value.

Postulate (v): The coordinates of any galaxy are random variables. Given the coordinates of the center of the cluster, the coordinates of a galaxy possess a conditional probability density represented by a continuous function $f(\eta)$ of argument η, the distance of the galaxy from its cluster center. Given the coordinates of all the cluster centers, the triplets of coordinates of the galaxies are all completely independent.

The above five postulates determine the general structure of the spatial distribution of galaxies studied. Among other results it was shown that no generality is lost in adopting the assumption that $\gamma(R)$ is a Poisson variable so that

$$G_\gamma(t \mid V) = e^{-\lambda V(1-t)} \tag{4}$$

where λ represents the expected number of cluster centers per unit volume.

In order to connect the properties of the model of the distribution of galaxies in space with what may actually be observed, it was necessary to adopt still another postulate.

Postulate (vi): Given the distance ξ of a galaxy from the observer and given a fixed observational setup, characterized by the limiting apparent magnitude m_1, there is a function $\theta(m_1, \xi)$ that represents the probability that the galaxy in question will be visible on a properly oriented photograph. When the distances of several galaxies and the limiting apparent magnitudes of the plates are fixed, then the visibility of one galaxy is independent of that of any other.

In a subsequent paper,[2] to be described as Paper II, joint with C. D. Shane, the above general model was specialized by postulating that the universe is static, by assuming (4) and that the unknown function $f(\eta)$ can be approximated by, say

$$f(\eta \mid \sigma) = \left(\frac{1}{\sigma\sqrt{2\pi}}\right)^3 e^{-\eta^2/2\sigma^2} \tag{5}$$

where $\sigma > 0$ is an adjustable parameter. Also, a definite form was adopted for $\theta(m_1, \xi)$ resulting from the studies of E. Hubble and M. Humason. Finally, at the end of the paper it was tentatively assumed that the distribution of $\nu - 1$ might be approximated by a negative binomial so that

$$G_\nu(t) = (1 + \beta - \beta t)^{-\alpha} \tag{6}$$

with $\alpha > 0$ and $\beta > 0$.

Using the observational data of Shane and Wirtanen[3] collected at the Lick Observatory, tentative estimates were obtained for all the parameters intervening in the specialized model. Unfortunately, these estimates depend very much upon the values of the constants determining the probability $\theta(m_1, \xi)$ which are only vaguely known. As a result, instead of single estimates of the parameters, there is a whole region of plausible combinations of values of these estimates.

In a third paper[4] of the same series, to be referred to as Paper III, by E. L. Scott, C. D. Shane, and M. D. Swanson, the consequences of adopting the numerical values

$$\lambda = 1.6 \times 10^{-19} \text{ cluster centers per cubic parsec}$$
$$\sigma = 5.0 \times 10^{5} \text{ parsecs}$$
$$\alpha = 1.00, \quad \beta = 91.5$$

were closely examined. It was found that the value of σ was probably overestimated and that, perhaps, $\sigma = 2.5 \times 10^{5}$ is closer to reality. On the whole, however, it was found that the implications of the specialized model of Paper II are in good agreement with the observations.

It will be noticed that the original model is capable of representing a great variety of distributions. On the one extreme, there is the uniform (Poisson) distribution of galaxies. This may be obtained from the model by assuming that $\nu = 1$ for all clusters and that γ follows a Poisson law. On the other extreme, the model implies the possibility that galaxies occur in compact clusters isolated from each other by great gaps of empty space. Finally, the possibilities covered in the model include distributions of intermediate types, that may perhaps be described intuitively as representing a continuous field of galaxies with smaller and larger local concentrations. The first of these possibilities is flatly rejected by the observations,[5, 6] so that it must be taken for granted that the distribution of galaxies in space is not statistically uniform. Also, several large clusters are now believed identified and are studied *per se*. However, information appears to be lacking regarding the last two possibilities mentioned, namely, whether such clusters as exist are separate isolated entities or interlock with each other and should be considered as local condensations in the generally uninterrupted field of galaxies.

Stated in this form, the question is phenomenal rather than mathe-

matical in character. The purpose of the present paper is to report on the authors' results that include the translation of the above question into mathematical terms, the deduction of certain formulae meant to answer the mathematical problem thus obtained, and numerical results based on the specialized model of Paper II and the estimates of the parameters reproduced above.

2. *Penetration of One Cluster by Galaxies from Other Clusters.*—Consider a particular cluster Γ_1 of galaxies and denote by C_1 its center. Also let ν_1 be the (random) number of galaxies belonging to Γ_1. These galaxies will be numbered in the order of their distance from C_1, beginning with the most distant galaxy. Let η_k stand for the distance between C_1 and the kth galaxy.

In accordance with the model of Paper I, the distance η_k is a random variable. The symbol $S(x)$ will be defined for $x \geqq 0$ to represent the sphere of radius x centered at C_1.

Now consider an arbitrary cluster Γ_2, other than Γ_1, and let ν_2 be the (random) number of galaxies it contains. If a galaxy of the cluster Γ_2 is located within $S(\eta_k)$, then we shall say that this galaxy penetrates Γ_1 to the depth k. If every one of the ν_2 galaxies of Γ_2 penetrates Γ_1 to the depth k, then we shall say that the cluster Γ_2 is imbedded in Γ_1 to the depth k.

Our present probabilistic treatment of the phenomenal problem of the interlocking of clusters of galaxies consists in computing the probabilities that the cluster Γ_1 will be penetrated by galaxies from other clusters and of probabilities that other clusters will be imbedded in Γ_1. The probabilities of this kind are interesting in relation to various hypotheses regarding ν_1 and ν_2. For example, we may consider that a cluster of, say, five galaxies is not very interesting and may wish to confine our considerations to clusters Γ_1 containing at least a specified number N_1 of galaxies. Also, it may be interesting to make similar restrictions regarding the size of the cluster Γ_2. For example, one may ask how frequently a cluster Γ_1 containing N_1 or more galaxies will have imbedded a specified number of small clusters Γ_2, each with $\nu_2 \leqq N_2$ galaxies. Alternatively, one may be interested to know how frequently a substantial cluster Γ_1 (with $\nu_1 \geqq N_1$ galaxies) is penetrated by galaxies from other substantial clusters, say with $\nu_2 \geqq N_2$ galaxies each.

Accordingly, the results given below are always based on the assumption that $\nu_1 \geqq N_1$ where N_1 is a number that may be specified arbitrarily. Also, the same results include the possibility of subjecting ν_2 to a restriction either $\nu_2 \leqq N_2$ or $\nu_2 \geqq N_2$. The letter H is used to denote any such hypothesis regarding ν_2. It appears in the symbols denoting the various probability generating functions. Also the symbol $P(H)$ is used to denote the probability, easily computed from $G_\nu(t)$, that the number ν of galaxies in a cluster will satisfy the hypothesis H.

The proofs of the formulae given below are somewhat long and will be published elsewhere.

3. Penetration of Γ_1 by Galaxies from the Nearest Cluster.—Given any two clusters of galaxies Γ' and Γ'' with centers at C' and C'', the term distance between Γ' and Γ'' will be understood to mean the distance between the points C' and C''. With this terminology, let Γ^* be the cluster that is nearest to Γ_1. Let ν^* be the number of galaxies belonging to Γ^*.

The subject of our interest in this section is the random variable $X(k)$ defined for $1 \leqq k \leqq \nu_1$ as the number of galaxies of the cluster Γ^* that penetrate Γ_1 to the depth k. The result given below represents the conditional probability generating function of $X(k)$, given that $\nu_1 \geqq N_1 \geqq 1$ and given that ν^* satisfies the hypothesis H. For $1 \leqq k \leqq N_1$ we have

$$G_{X(k)}[t \mid (\nu_1 \geqq N_1)H] = \int_0^1 \{\Phi(v, k, N_1) \int_0^1 G_\nu(\tau \mid H)du\}dv \quad (7)$$

where

$$\Phi(v, k, N_1) = \frac{1}{(k-1)!\,P\{\nu_1 \geqq N_1\}} (1-v)^{k-1} \sum_{n=N_1}^{\infty} \frac{n!}{(n-k)!} v^{n-k} P\{\nu_1 = n\} \quad (8)$$

$$\tau = 1 - (1-t)\,F[x(u), y(v)] \quad (9)$$

$$F(\xi, \eta) = \iint_{S(\eta)} \int f\{[(x-\xi)^2 + y^2 + z^2]^{1/2}\}\,dx\,dy\,dz \quad (10)$$

with $x(u)$ and $y(v)$ defined for $0 \leqq u, v < 1$ by

$$x(u) = \sqrt[3]{\frac{-3 \log (1-u)}{4\pi\lambda P(H)}} \quad (11)$$

and

$$4\pi \int_0^{y(v)} x^2 f(x)\,dx \equiv v. \quad (12)$$

The functions defined in (8), (10), and (12) will be used in Sections 4 and 5.

In order to compute (7) numerically, it is sufficient to know the functions f and G_ν. Obviously, if one substitutes $N_1 = k$ and interprets H as $\nu^* \geqq 1$, then the conditional probability generating function (7) becomes the unconditional probability generating function of $X(k)$. Formula (7) contains the answers to such questions as the following: How frequently is a cluster Γ_1, of at least $N_1 = 100$ galaxies, penetrated to the depth $k = 5$ by $X(5)$ galaxies all of which belong to Γ^* which is the nearest of all other clusters containing at least $N_2 = 50$ galaxies? Of particular interest is the question: How frequently is Γ_1 not penetrated by any of the galaxies of the cluster Γ^*? The answer is obtained by substituting $k = 1$ and $t = 0$ into formula (7).

While formula (7) does provide interesting information regarding the interlocking of clusters, this information is a little one-sided. The reason is that the given cluster Γ_1 may be isolated from the nearest cluster Γ^* (so that $X(1) = 0$) but yet have penetrations from more distant clusters. This problem is dealt with in the next section.

4. The Number of Clusters Which Have Some Galaxies Penetrating a Given Cluster.—As formerly, let k be a positive integer not exceeding the number ν_1 of galaxies belonging to a cluster Γ_1. The letter ν_2 will be used to denote the number of galaxies of a cluster Γ_2 other than Γ_1. In this section we consider $Y(k)$ defined as the number of clusters Γ_2 that have at least one galaxy penetrating Γ_1 to the depth k. Thus, if $Y(1) = 0$ then the cluster Γ_1 is completely isolated.

The following formula gives the conditional probability generating function of $Y(k)$, given that $\nu_1 \geqq N_1 \geqq k$ and given that ν_2 satisfies the hypothesis H.

$$G_{Y(k)}[t \,|\, (\nu_1 \geqq N_1)H] = \int_0^1 e^{-\lambda(1-t)I(v,\,H)}\, \Phi(v, k, N_1)\, dv \qquad (13)$$

where

$$I(v, H) = 4\pi P(H) \int_0^\infty \xi^2 \{1 - G_\nu[1 - F(\xi, y(v)) \,|\, H]\}\, d\xi. \qquad (14)$$

The probability that a cluster containing at least N_1 galaxies will be completely isolated from all those clusters in which the number ν_2 of galaxies satisfies the hypothesis H is obtained from (13) by substituting $k = 1$ and $t = 0$.

5. Number of Clusters of Galaxies That Are Imbedded in a Given Cluster.—In Sections 3 and 4 we considered the problem of penetration of a cluster by galaxies belonging to other clusters. In the present section we are concerned with a random variable $Z(k)$ characterizing the frequency with which a given cluster Γ_1 includes within its bounds other clusters, with all their galaxies. Physically, these clusters imbedded in Γ_1 would probably be considered as irregularities in the structure of Γ_1.

The random variable $Z(k)$ is defined as the number of clusters Γ_2, other than Γ_1, that are imbedded in Γ_1 to the depth k. The conditional probability generating function of $Z(k)$, given that the cluster Γ_1 contains at least $\nu_1 \geqq N_1 \geqq k$ galaxies and given that the number ν_2 of galaxies in the cluster Γ_2 satisfies the hypothesis H, is given by the formula

$$G_{Z(k)}[t \,|\, (\nu_1 \geqq N_1)H] = \int^1 e^{-\lambda(1-t)J(v,\,H)}\, \Phi\,(v, k, N_1)\, dv \qquad (15)$$

where

$$J(v, H) = 4\pi \int_0^\infty \xi^2 G_\nu[F(\xi, y(v)) \,|\, H]\, d\xi. \qquad (16)$$

6. Some Numerical Results.—As mentioned in the Introduction, the available estimates of the parameters λ, σ, α, and β involved in the

model of Paper II are still very vague and the detailed numerical study of the actual distribution of galaxies using the formulae given above must be postponed until these constants are known with reasonable precision. Nevertheless, it appears interesting to use such estimates as are now available in order to gain some idea as to the degree of mutual interlocking of clusters of galaxies. With this in mind, the above general formulae were specialized by substituting (5) and (6). Finally, using the constants λ, α, and β as given in the Introduction and also two alternative values of $\sigma = 5 \times 10^4$ and $\sigma = 2.5 \times 10^5$ parsecs, the following results were obtained. All of them refer to the hypothesis that $\nu_1 \geqq 1$ and $\nu_2 \geqq 1$.

	$\sigma = 5 \times 10^5$	$\sigma = 2.5 \times 10^5$
$P\{X(1) = 0\}$	0.0098	0.25
$E[X(1)]$	69.5	27.4
$P\{Y(1) = 0\}$	0.0017	0.20
$E[Y(1)]$	14.	1.8

It will be seen that the conclusion regarding the general appearance of the distribution of galaxies in space depends very much on the value of σ. If the larger of the two values is close to reality, then only about 1 per cent of all clusters are isolated from the nearest cluster and the average number of galaxies of the nearest cluster that penetrate the given cluster is 69.5. Also, the percentage of all clusters that are not penetrated by galaxies from any other cluster is only 0.17 per cent. Finally, the average number of clusters that have galaxies penetrating the given cluster is 14. It follows that, with the particular estimates of the parameters, a cluster of galaxies as a physical entity is, probably, a rarity. Thus, if one attempts to build a dynamic model of the structure of the universe, then this model should consider all the galaxies as forming a single complicated system, rather than many separated systems each represented by a cluster.

On the other hand, if $\sigma = 5 \times 10^5$ is an overestimate (as indicated in Paper III) and $\sigma = 2.5 \times 10^5$ is closer to actual fact, then the above figures indicate that a substantial proportion of clusters are isolated from the others and, probably, could be considered as separate dynamic systems.

Acknowledgment.—It is a pleasure to record the authors' indebtedness to Mrs. Jeanne L. Lovasich for the careful calculations connected with the present paper.

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THE INFLUENCE OF TEMPFRATURE UPON THE INACTIVATION OF A BACTERIAL VIRUS BY X-RAYS

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A discussion of the nature of the mechanisms of radiation damage in living systems requires distinction between so-called direct effects on sensitive sites or "targets," and indirect effects, by which the biological action is accomplished via some intermediate entity in the system such as a free radical produced directly by the radiation. One of the methods used to distinguish direct from indirect action consists in measuring radiosensitivity of the system while suspended in liquid media of different composition. In this manner one presumably can estimate the amount of direct action by comparing results obtained in pure water with those in some organic medium which, having high affinity for very reactive radiation-produced substances, should protect the cells from these intermediate substances. Such an approach has been used by a number of authors, a recent instance being Watson's study of radiation effects in the bacteriophages of *Escherichia coli*.[1, 2]

It cannot be presumed, however, that changes in the outside environment allow discrimination of direct and indirect effects within the cell itself, or even within the virus particle, and for distinction on a subcellular and subparticle scale other methods have to be employed.

One method is a study of the effect of temperature on radiosensitivity. Above the very low temperature region temperature changes probably should not affect ionization and direct inactivation of a vital target, but temperature changes should affect the activity of any intermediate substance that affects secondarily the sensitive site, either by changing diffusion rates or by affecting reactivity itself, or by both methods.

In this investigation we have studied the effects of widely separated temperatures during x-irradiation on survival of the T1 bacteriophage of *E. coli*. These particles appear to be inactivated by single ionizations and they can be dried and carried to very low temperatures with little inactivation. In this dried state one is dealing with effects within the particle itself, and any separation of effect brought about by the temperature differences occurs within the virus.

A preliminary report has appeared previously.[3]

Materials and Methods.—Suspensions in broth of T1 phage were dried in aluminum sample cups, irradiated in vacuo at controlled temperatures,

and assayed for survival on *E. coli*, strain B. The method of preparation involved distribution of 50 λ or 100 λ aliquots of broth suspensions of approximately 5×10^9 particles per ml. as a drop at the bottom of each cup. The cup was an Aloe-Willett cap of 22 mm. outside diameter, modified at the bottom by a depression 14 mm. in diameter and 1 mm. in depth. The depression defined the maximum diameter of the drop. Such preparations were dried from the liquid state in a 160-mm. vacuum top desiccator, by evacuation with a Duo Seal Vacuum pump in a cold-room at 0–3°C. Evacuation was controlled to proceed in slow stages, with 3–4 hrs. elapsing in the first stage before the pressure was allowed to drop below 5 mm. Hg. Inactivation and loss associated with boiling in early stages was thus avoided. After the first stage, the pump was allowed to run continuously for 16–18 hrs., the pressure falling below 1 mm. Hg within the first hr. of this interval. This drying procedure resulted in less inactivation and less variability between replicate samples than any other method employed, including lyophilization. Nevertheless, the data from several experiments had to be rejected on account of excessive inactivation attributable to the drying process. In the four experiments reported here, survival through dehydration and rehydration was higher than 95%. The dried preparations were stored at 25°C., and were kept at atmospheric pressure except during irradiation and temperature exposure.

The irradiation apparatus included a 50-kv. Picker machine with a Machlett beryllium window tube operated at 40 ma. without additional filtration. A doughnut-shaped chamber with an internal bore of 20 mm. diameter was affixed to the head of the tube. At one end of the bore was the Be window and at the other end the sample cup, the irradiated sample being 10 cm. from the target of the tube. Evacuation of the chamber was effected through a side-arm connected to the bore and monitored by a manometer. Pressure was maintained at less than 0.1 mm. Hg throughout the exposure interval. Temperature at the bottom of the cup was monitored by an iron-constantan thermocouple located on its outer surface, while the entire sample cup was immersed in an appropriate isothermic medium within a Dewar flask. The isothermic media employed were liquid nitrogen (77°K.), solid carbon dioxide-acetone (176–180°K.), ice water (273°K.), and water (310°K.).

The dosimetry of the system employed has not yet been determined accurately. Variations in doses delivered were accomplished by varying the time of exposures, and all doses are expressed in terms of minutes. The best estimate of actual dosage is based on the manufacturer's estimate of approximately 62,000 r/min. at 10 cm. As noted below the results obtained agree very well with those of others.[4]

Immediately after irradiation, samples were restored to 25°C. and

atmospheric pressure. The phage particles were resuspended in broth, and dilution and plating were carried out in conventional fashion.[5] The maximum time lapse between irradiation and plating was 4 hrs., and replicate samples from the beginning or end of this interval indicated absence of a standing effect when broth is the suspending medium. Effects of temperature and pressure changes *per se*, if present, were too small to be detected beyond the survival limits of the controls. In each experiment an average of 3 sample cups per point was employed, and titers were calculated from plaque counts of 5 plates per cup.

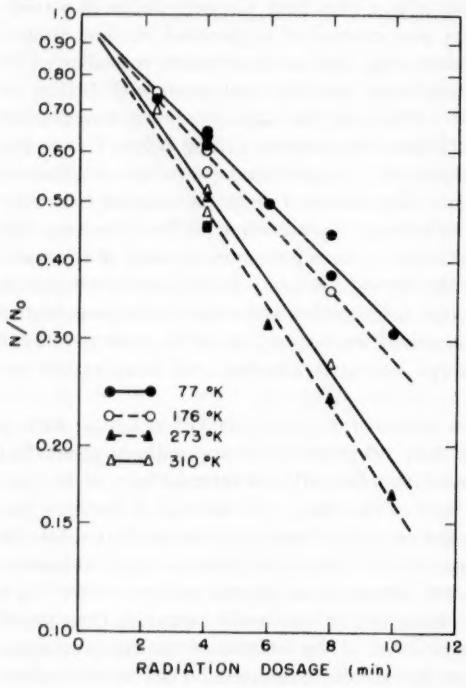

FIGURE 1

The relationship between survival of bacteriophage T1 dried from broth and relative dosage of x-rays at indicated temperatures. The solid rectangular point is at 300°K.

Results.—The relationship between treatment and survival at a particular temperature (Fig. 1) is considered exponential and the relation,

$$N/N_0 = e^{-k_T D} \quad (1)$$

appears to be valid, with dose (D) being in minutes of irradiation under the conditions described, and k_T being the dose inactivation constant in reciprocal minutes at the absolute temperature. The numerical value of k_T ($\pm$ its standard error) at each temperature was estimated by a least squares approximation for each exponential line to be as follows: at 77°K. it is 0.115 ± 0.007; at 176°K. it is 0.128 ± 0.015; at 273°K. it is 0.180 ± 0.010; and at 310°K. it is 0.166 ± 0.014.

These values are plotted against temperature in figure 2. The type of response is not determined by the points, but assuming a linear relationship, we calculate that

$$k_T = 0.00027T + 0.091. \quad (2)$$

When this is substituted into expression (1), the survival of dried T1 phage is empirically described as

$$N/N_0 = e^{-D(0.091+0.00027T)} \quad (3)$$

when treated and measured under the conditions described herein.

It is important to note that this expression is known to be valid only for temperatures below 37°C. Even at 37°C. there is some thermal inactivation with time. For instance, at 35°C., T1 phage in our laboratory dried from broth is inactivated by about 10% over 24 hr.; at higher

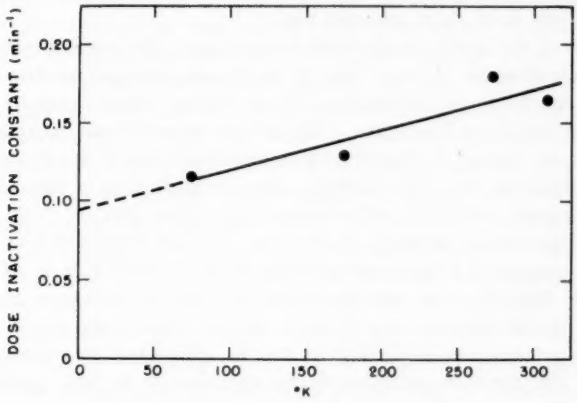

FIGURE 2

The relationship between the dose inactivation constant and temperature for dried coliphage T1.

temperatures more rapid loss is certainly to be expected.[6] Preparations held at 25°C. for 24 hrs. and at 35°C. for 4 hrs. were indistinguishable from others held at 0–5°C., for the same lengths of time. At the two higher temperatures, manipulations, as indicated above, were rapid enough to obviate the added complication of simultaneous inactivation from two treatments, heat in the higher ranges and x-rays, and no consideration is being given here to temperature results above 310°K.

The general empirical expression relating survival with dose and temperature indicates that at absolute zero there may be a real dose inactivation constant (the intercept of the line in Fig. 2). The latter extension has not, of course, been tested. Indeed the authors do not consider that 77°K. is close enough to predict with certainty that inactivation will proceed at absolute zero at the rate indicated, and there is need for tests at temperatures much closer to zero than it has been possible to make to date. However, the intercept indicated is a measure of the tempera-

ture-independent radiation effect, and if it exists it alone is a measure of sizes or cross-sections of targets that can be believed to be *directly* affected by radiations.

Discussion.—Adams and Pollard[4] report no apparent change in inactivation volume at temperatures between 203°K. and 310°K., but it should be noted that the spread of the points in their low temperature region together with the narrow temperature range tested mask an effect of the magnitude reported in this paper. The temperature effects on radiation sensitivity reported by them occur above 40°C., and being strikingly different in magnitude should probably be considered different in mechanism from those reported here.

In view of the small change with temperature, the possibility was considered whether the physical size of the target decreases as the particle contracts in the lower temperatures to an extent which accounts for the lowered efficiency of the x-rays. We are not aware of any measurements of change in volume of phage with temperature, but if we choose as an outside condition one of the highest values for coefficient of cubical expansion of organic materials with temperature cited (tar = $6\text{–}8 \times 10^{-4}$ volume/unit volume/degree C.), over the range of 77 to 310 we calculate a volume increase for this material of only 18.6% ($233 \times 8 \times 10^{-4}$). It is unlikely that the virus particle physically expands with increasing temperature to this extent. Yet relative "target" size of the phage particle at 77°K. as compared with that at 310°K. calculated by comparing e^{-1} doses at the two temperatures (from equation 3) is 56% greater (8.9 min./5.7 min. = 1.56), nearly three times that which can be accounted for in terms of cubical expansion. Therefore, even if cubical expansion of the particle contributes to the effect, it seems unlikely that it is more than one of the minor reasons why a temperature rise results in increased radiation efficiency.

An analysis of the change with temperature in rate of inactivation by x-rays in standard chemical kinetic terms shows that the usual test for simple kinetics (logarithm of rate against reciprocal of temperature) using the Arrhenius formulation does not yield a straight line, nor, indeed, is such a result necessarily to be expected. Furthermore, in the absence of much more elaborate evidence, a thermodynamic and kinetic explanation critically demonstrating a mechanism or mechanisms is impossible.

However, a tentative model in kinetic terms is possible. Suppose that the inactivation constant is composed of two components, one temperature independent and one temperature dependent. The constant observed at any given temperature should, then, be separable into these two components by subtraction, and the value which when subtracted leaves residues which are in a first order Arrhenius relation ($k' = e^{-\Delta H/RT}$) with temperature is, according to this model, the inactivation constant of the

temperature independent portion. It was found that a straight line relation is approached as the value of the temperature independent portion of the constant approaches the value of the total inactivation constant at 77°K., and the "activation energy" indicated by the residues obtained in this way is about 1100 calories.

The temperature independent part of the inactivation constant may be thought of as the rate at which x-rays inactivate the virus particle by affecting directly the sensitive sites. But there are several conceivable meanings that could be attached to the temperature dependent portion of the inactivation constant. For instance, the ΔH of about 1100 calories or some portion of it can represent the activation energy of some reactive compounds produced by radiation. The value estimated here is not out of line with the estimated values for free radicals. Or, the estimated constant may describe, at least in part, the effects of temperature on some diffusion mechanism. That such exist in the solid state is indicated by Mayburg and Lawrence[7] who describe changes in conductivity of polyethylene after γ-irradiation at different temperatures and who calculate Q values as low as 3000 cal., representing to them the control by temperature of the migration of protons in this solid system. Other similar studies have been made on the relation between temperature and the development of latent photographic images.[8] In biological systems a diffusion mechanism has been offered to explain the action of liquid nitrogen temperatures in modifying x-ray effects in plant seeds and pollen,[9] with effectiveness of a given dose being reduced by 80% at the low temperatures.

The meaning of this interpretation in regard to exponential survival curves is that while the "single hit" interpretation is still allowed, the hit may occur very close to but not necessarily within the sensitive site itself. The intermediate substances formed are very reactive chemically as indicated by the low estimate of over-all activation energy, can migrate but short distances before reacting and removing themselves from the system, and, therefore, have very short lives. The consequence is an appearance at ordinary temperatures of temperature independence and direct action.

The data of course do not require the interpretation described above. Indeed, the model suggested by Adams and Pollard[4] which demands the simultaneous breakage of several critical bonds can conceivably be made to fit. Furthermore, the possibility of competitive reactions with different energy requirements, recombinations within vital targets, or several combinations of these with the diffusion model are not eliminated. Our statement of the energy requirement is an over-all description that does not separate one possibility from any other.

Even without a clear physicochemical explanation, these results impose

a refinement on all considerations of target size in T1 coliphage deduced from x-irradiation data. Using our inactivation value for 310°K., and the rated dosage of the 50 kv.p. x-ray machine, we calculate the sensitive volume of T1 in our laboratory to be 2×10^{-18} cm.^{3}, which agrees with the value reported by Adams and Pollard[4] for the range 203° to 313°K. The results here indicate, however, that the value at very low temperatures may be one-half of that indicated by measurements at room temperature, and any consideration of the structure of phage based on x-irradiation data is subject to this restriction.

Acknowledgment.—We wish to acknowledge valuable discussions with G. A. Sacher and G. R. Hennig of this laboratory.

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*ELECTRON MICROSCOPY OF THE NUCLEIC ACID RELEASED FROM INDIVIDUAL BACTERIOPHAGE PARTICLES**

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It has been known for some years[1] that certain bacterial viruses can be "shocked" osmotically by rapid dilution into distilled water of a suspension of virus in a concentrated salt solution. As a result of this treatment the suspension loses most of its virus activity, and becomes extremely viscous. When the mixture is subjected to differential centrifugation, instead of the typical virus particles normally observable in the electron microscope[1] only flattened virus ghosts are found in the sediment. Herriott[2] has shown that these ghosts are free of the nucleic acid which makes up some 40% of the intact virus, and are composed of protein. The ghosts retain the origi-

nal ability of the virus to absorb onto, kill, and lyse the host bacterium but do not have the ability to reproduce. By means of studies in which the protein portion of the virus was labeled with radioactive sulfur and the nucleic acid portion with radioactive phosphorus, Hershey and Chase[3] have shown that the sulfur label does not enter the infected host, but remains attached to the cell wall, whereas the phosphorus label enters the cell. Since it has also been found that the sulfur-labeled protein can be removed from the infected bacterium after absorption without influencing reproduction, the presumption is that only the nucleic acid portion is responsible for the reorganization of the cell metabolic processes resulting in replication of the infecting virus. It cannot be said categorically that the nucleic acid moiety is absolutely protein-free—only that it contains no sulfur and no detectable protein by various tests—but the most obvious conclusion is that the genetic material of the virus is nucleic acid. From all appearances and from the above-mentioned work it also seems apparent that the protein portion left behind on infection is essentially the ghost seen when the phage is shocked osmotically. It can be concluded that the material released by shocking is primarily nucleic acid and that this is the genetic substance. In this paper this material will be called nucleic acid.

Discussion and Results.—Because of the great importance of this genetic substance it has seemed to us most attractive to examine the nucleic acid released from burst bacteriophages in the electron microscope. Our first experiments consisted of examining the supernatant fluid left after osmotically shocking and centrifuging a sample of purified bacteriophage, essentially by the methods previously used, but using in the original, hypertonic solution a completely volatile solute such as ammonium acetate or glycerol. We found that the preparation contained largely elongated filamentous material—what one might expect from the known properties of nucleic acid and previous electron micrographs.[4] These experiments, however, had the disadvantage that there was no way of identifying any given strands with any particular virus particle.

An obvious improvement would be to shock the virus particles *in situ* on the electron microscope screen. The techniques previously developed by Backus and Williams[5] for spraying minute droplets and by Williams[6] for freeze-drying biological specimens for the electron microscope seemed particularly well-suited for this purpose. Specimens in water sprayed in the usual way[5] and resprayed, before drying, with a concentrated glycerol solution showed a considerable proportion of shocked phage particles where sets of droplets overlapped. Stranded material was evident in the shocked specimens and completely absent in controls which had received a single spraying of either the virus suspension or the glycerol solution. Unfortunately, however, a glycerol-water solution cannot be sublimed at a practical rate at temperatures below $-60°C$. During sublimation the eutectic

concentration is inevitably reached and even at −60°C. the frozen material partially melts.

In the course of exploratory experiments on alternative methods we found, somewhat surprisingly, that if a sample of virus sprayed from distilled water and dried on the electron microscope screen, either air-dried or

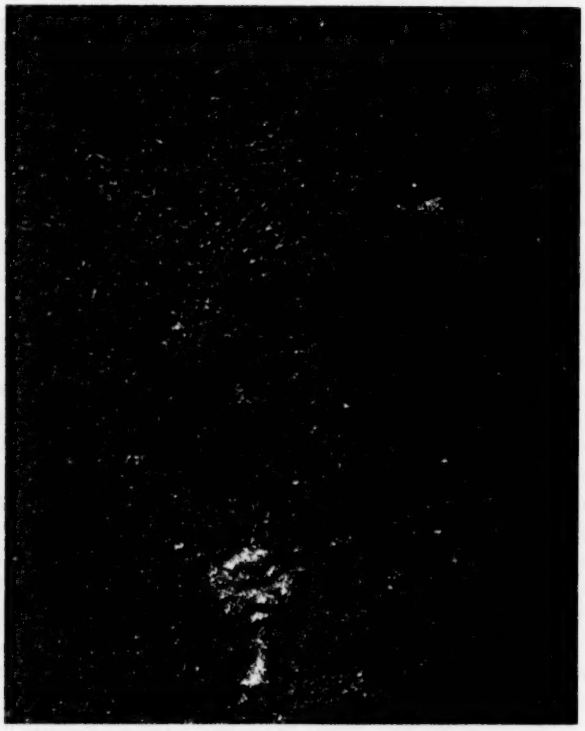

FIGURE 1

Electron micrograph of a broken T6 bacteriophage particle. The empty membrane of the head, and the intact tail, are shown at the bottom of the picture. The fine strands of deoxyribonucleic acid are seen spread out in an approximately circular array over most of the micrograph. Shadowed with uranium at $\tan^{-1} 1/4$. ×94,000.

frozen-dried, is rewetted by a second spraying of distilled water or by condensed water vapor, the particles take on the flattened, eviscerated appearance typical of osmotically shocked phages. In our opinion the phenomenon is not an osmotic effect, but is induced by the forces of surface tension which act on the virus particles on the microscope screen as a drop dries or forms.

This observation made possible a very simple technique for breaking open phages. In the current experiments, the virus particles are sprayed from water upon a collodion-covered electron microscope screen, are frozen-dried in the usual way,[6] and are then exposed to moist air, which enters the sublimation chamber with the specimen at −20°C. The phage particles on the screen serve as nuclei for condensation of the supercooled water vapor and are broken open by surface tension. The sublimation chamber is then cooled to freeze the condensed water droplets and the frozen droplets vacuum-sublimed. The virus concentration is so adjusted that each condensation droplet contains only a few particles. An isolated phage ghost is thus easily identified with the released nuclei acid.

The results of such an experiment are shown in figure 1. At the lower left of the picture the typical phage ghost can be seen and above it a collection of the nucleic acid which has unraveled almost completely into individual fibrils. The strands are so fine that they are discernible and measurable only because of the uranium shadows. Their diameter can be approximated by measuring the width of the shadows, and is estimated to be 20 ± 5 Å. This value agrees excellently with the known diameter of elementary deoxyribose nucleic acid fibrils as determined by x-rays and other methods.[7, 4] The number of strands is most difficult to determine because of their very small diameter and their baffling interweaving. At the present time no significance can be attached to any of the details of structure of the fibrils.

Summary.—A method has been devised whereby bacterial viruses can be broken open *in situ* on an electron microscope screen in such a way that the contents of the phage are released and can be identified with a given virus particle. The fibrillar strands which can be presumed to be the nucleic acid or chromosomal material of the virus, have a diameter of 20 Å, a value which agrees closely with that obtained by other methods for the elementary fibrils of deoxyribose nucleic acid.

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TRANSFER OF C^{14} BY LUPINE MITOCHONDRIA THROUGH REACTIONS OF THE TRICARBOXYLIC ACID CYCLE*

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Many observations of the behavior of organic acids in plants are explicable in terms of the functioning of a tricarboxylic acid cycle. Generally the most abundant organic acids in plant tissues are malic, citric, and oxalic acids. Oxalate is relatively inert and appears to be an end-product rather than an active metabolite. As malate and citrate show marked quantitative responses to changes in the physical environment of the plant and behave otherwise as active metabolites, it has been proposed that several of their conversions are mediated through a tricarboxylic acid cycle. For example, Pucher, Wakeman, and Vickery[1] have shown that excised tobacco leaves in the dark lose malate and accumulate citrate, and Zbinovsky and Burris[2] have found that tobacco leaves infiltrated with C^{14}-labeled malate produce labeled citrate.

Within the past few years, evidence has accumulated in support of the existence of the tricarboxylic acid cycle in plants, and the recent observations of Millerd *et al.*,[3, 4] and Bonner and Millerd[5] compel one to conclude that the cycle is operative in certain plant tissues. These workers have demonstrated the oxidation of the acids of the tricarboxylic acid cycle and the oxidative phosphorylation by mitochondria from mung bean seedlings. In the experiments reported here, the presence of the cycle is indicated by transfer of radiocarbon from pyruvate-2-C^{14} into the intermediates of the tricarboxylic acid cycle.

Methods and Materials.—Preparations of particles, identified as mitochondria by morphology and affinity for janus green, were obtained from the cotyledons of etiolated white lupine seedlings in a manner similar to that described by Millerd *et al.*[3] The seeds (*Lupinus albus*) were soaked for 12 hours in distilled water prior to incubation in moist sand at 28°C. The cotyledons of seven-day-old seedlings were removed, and 70 g. of cotyledons, 70 ml. of 0.2 *M* sucrose solution and enough solid K_2CO_3 to bring the final pH of the brei to 6.5–7.0 were ground together with quartz sand in a mortar. All operations preceding the measurement of activity were performed at 0°C. and solutions and utensils were prechilled to this temperature. The brei was strained through cheese cloth and centrifuged at 1000× G for 10 minutes to remove nuclei and cell debris. The supernatant was then centrifuged at 20,000× G for 10 minutes, and the yellow pellet obtained was resuspended in 0.2 *M* sucrose and recentrifuged at 20,000× G for 15 minutes. This yellow sediment resuspended in 20–25

ml. of 0.2 *M* sucrose was used as the enzyme preparation. The suspension contained 0.5–1.0 mg. N/ml. and constituted half the volume of the reactants in the manometric flask. All of the experiments were performed at 30°C. in conventional Warburg manometric apparatus.

Pyruvate, oxalacetate, α-ketoglutarate, and oxalosuccinate were separated as their 2,4-dinitrophenylhydrazones on silica gel columns by a method similar to that employed by Sanger[6] for the separation of the 2, 4-dinitro-N-phenyl derivatives of amino acids. The columns were prepared by uniformly mixing 1.4 ml. of an aqueous buffer solution, which was equilibrated against the organic solvent used for developing the column, with 2.0 g. of silica gel (Mallinckrodt, 100 mesh, analytical reagent silicic acid). About 25 ml. of the organic phase was mixed with the silica gel, and the resulting slurry was poured into a 1 cm. diameter column. The solvent systems used were 5 per cent butanol, 95 per cent chloroform (all solvents given as volume per cent) equilibrated against a 1.0 *M* potassium phosphate buffer of pH 6.8; 15 per cent butanol, 85 per cent chloroform equilibrated against a 1.0 *M* potassium formate buffer of pH 3.8; and 7 per cent butanol, 93 per cent chloroform equilibrated against a 1.0 *M* potassium phosphate buffer of pH 2.0.

The hydrazones of the keto acids were prepared by mixing an excess of a 0.1 per cent solution of 2,4-dinitrophenylhydrazine in 2.0 *N* HCl with the free acids or with the preparation to be determined according to the method of LePage.[7] The hydrazones then were extracted with ether in a Kutscher-Steudel extractor for 1 hour; the ether was removed under vacuum, and the residue was taken up in 0.5–1.0 ml. of the organic developing solvent and was placed on the appropriate column. For separation of the oxalosuccinate derivative, the 7 per cent butanol, 93 per cent chloroform solvent was employed. Derivatives of oxalacetate and α-ketoglutarate were separated with the 15 per cent butanol, 85 per cent chloroform solvent, and the pyruvate 2,4-dinitrophenylhydrazone was recovered with 95 per cent chloroform, 5 per cent butanol as solvent. In these systems, the pyruvate and oxalacetate 2,4-dinitrophenylhydrazones were resolved into their geometric isomers. Recoveries of 98–101 per cent were obtained from known pyruvate, oxalacetate, and α-ketoglutarate when about 100 μg. quantities of these acids were determined. The quantity of each 2,4-dinitrophenylhydrazone was measured in a 1 cm. cell in the solvent in which it was eluted from the column; the quantitative observations were made in a Beckman model DU spectrophotometer at the wave length of maximum absorption for each derivative (range 340 to 365 mμ).

Experimental Results and Discussion.—As shown in figure 1, the addition of magnesium ions and ATP (adenosine triphosphate) stimulated the oxidation of pyruvate plus malate by mitochondria, but Armour Labora-

tories' "liver coenzyme concentrate" stimulated much more. Figure 2 indicates that the addition of DPN of greater than 95 per cent purity stimulated the oxidation of malate plus pyruvate. Armour Laboratories' "liver coenzyme concentrate," which contains DPN, TPN (di- and triphosphopyridine nucleotides) and CoA (coenzyme A) and which stimulated the uptake of oxygen, was added routinely in later experiments.

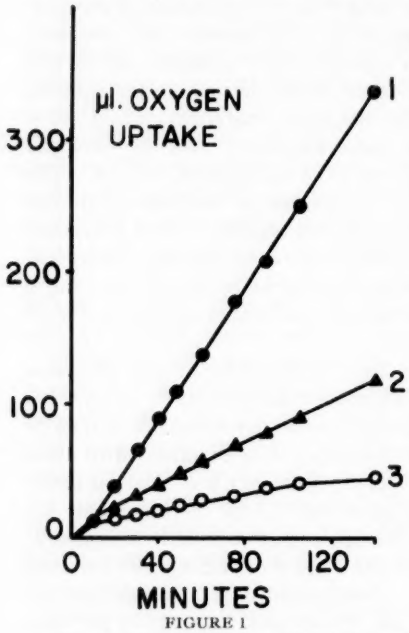

FIGURE 1

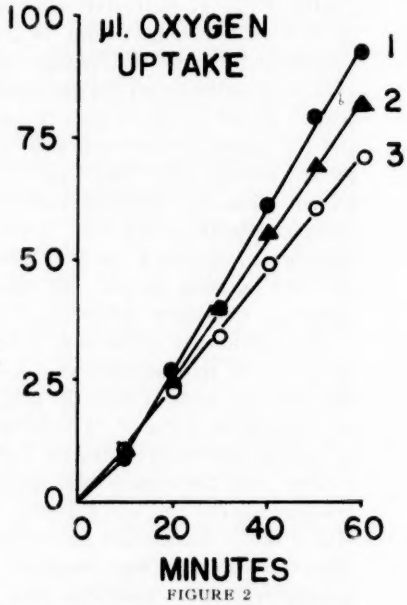

FIGURE 2

Fig. 1—The influence of ATP (adenosine triphosphate) plus $MgSO_4$, and of CoA (coenzyme A) on the oxidation of pyruvate plus malate by lupine mitochondria. Each flask contained mitochondria, 50 µm potassium phosphate buffer pH 7.0, 20 µm L-malate, and 20 µm pyruvate; final volume 2.0 ml. Temperature, 30°C. (*1*) 1 mg. "liver coenzyme concentrate" added. (*2*) 2 µm $MgSO_4$ plus 0.2 µm ATP. (*3*) Nothing added. Fig. 2—The influence of diphosphopyridine nucleotide and coenzyme A on the oxidation of pyruvate plus malate by lupine mitochondria. Substrates and buffers supplied as in figure 1. (*1*) 2.5 mg. "liver coenzyme concentrate" added. (*2*) 0.25 or 0.05 mg. DPN added. (*3*) 0.25 mg. "liver coenzyme concentrate" added.

In contrast to the preparations of Millerd *et al.*,[3] it was unnecessary to grind the lupine tissue in phosphate buffer; added cytochrome-c did not influence the uptake of oxygen. The rates of oxidation of pyruvate in the presence of L-malate or oxalacetate are shown in figure 3. As oxalacetate inhibited oxygen uptake for the first 30 minutes, L-malate was used subsequently as the "sparking" acid.

Manometric measurements indicated that malate and pyruvate in combination were oxidized at a more rapid rate by the mitochondrial preparations than the sum of their rates singly. These preparations thus seemed to provide an enzymatic system suitable for testing incorporation of a tracer into intermediates of the tricarboxylic acid cycle. Tests were made by adding to a mitochondrial preparation 200 μm of pyruvate-2-C^{14}, 200 μm of L-malate and 200 μm of another acid of the cycle to serve as a trapping acid. During an hour's incubation at 30°C. in a 125-ml. Warburg flask, the trapping acid equilibrated with the same acid as it was formed enzymatically. Then the trapping acid and any other acids present in sufficient quantity were isolated by partition chromotography on silica gel[8] and were analyzed for their content of C^{14}.

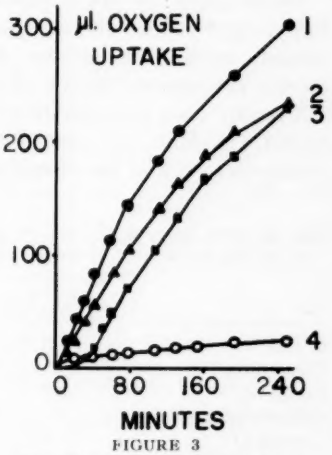

FIGURE 3

The effect of malate and oxalacetate on the oxidation of pyruvate by lupine mitochrondria. All flasks contained 50 μm potassium phosphate buffer pH 7.0, 1 mg. "liver coenzyme concentrate" and mitochondria in a final volume of 2.0 ml. (*1*) 10 μm malate plus 20 μm pyruvate. (*2*) 10 μm malate. (*3*) 10 μm oxalacetate plus 20 μm pyruvate. (*4*) 10 μm oxalacetate or 20 μm pyruvate alone.

Data presented in table 1 show that there is an appreciable incorporation of C^{14} from pyruvate-2-C^{14} into every acid of the tricarboxylic acid cycle; this indicates the participation of each acid in the cyclic oxidation of pyruvate. In the experiment in which malate was supplied, there was a net accumulation of 21 μm of citrate. The specific activity of the citrate was 32.6 counts $\times$ min.$^{-1}$ $\times$ μm^{-1} and that of the original pyruvate 31.5 counts $\times$ min.$^{-1}$ $\times$ μm^{-1}. Thus it is evident that the citrate arose directly from pyruvate and malate, since the molar specific activities of citrate and pyruvate were essentially the same. The substantial accumulation of citrate suggests the presence of an active condensing enzyme which can effect a condensation of acetyl CoA and oxalacetate as occurs in isolated animal tissues.[9] That the subsequent oxidation of pyruvate proceeds via the other acids of the tricarboxylic acid cycle is shown by the accumulation of appreciable amounts of C^{14} into every acid of the cycle. The variability of the amount of C^{14} found in individual acids after each experiment probably resulted from variability in recovery as well as differences in the activity of specific preparations. The accumulation of C^{14} in the respired CO_2 shows that the oxidation of a portion of the

pyruvate is complete. The small percentage accumulation of C^{14} in the respired CO_2 in these experiments can be attributed to the effective trapping of the C^{14} by the pools of acids added as sparker and trapping agents. This small accumulation of $C^{14}O_2$ also constitutes evidence for the asymmetrical synthesis and oxidation of citric acid in plant tissues, a process previously demonstrated in animal tissue.[10] As much C^{14} should have appeared in the CO_2 as appeared in succinate, fumarate, malate, and oxalacetate if the conversion had been symmetrical.

These results, in conjunction with earlier evidence that mitochondria can readily oxidize the intermediates of the tricarboxylic acid cycle, strongly support the premise that pyruvate oxidation by lupine mitocondria proceeds via the conventional tricarboxylic acid cycle. As plants can readily form pyruvate from carbohydrates, it is evident that carbohydrates in plants can be completely oxidized to CO_2 and H_2O in a manner analogous to that of the animal cell. How widespread and active the tri-

TABLE 1

THE ACCUMULATION OF C^{14} IN CO_2 AND ACIDS OF THE TRICARBOXYLIC ACID CYCLE FROM PYRUVATE-2-C^{14} SUPPLIED TO PREPARATIONS OF LUPINE MITOCHONDRIA

TRAPPING ACID ADDED	PER CENT C^{14} FOUND IN TRAPPING ACID RECOVERED	PER CENT C^{14} FOUND IN CO_2 FORMED	PER CENT C^{14} FOUND IN CITRATE FORMED	PER CENT C^{14} FOUND IN MALATE RECOVERED
Citrate	6.5	1.0	..	0.7
Cis-aconitate	1.5	0.5	30.2	0.6
Isocitrate	2.2	1.0	49.0	5.0
Oxalosuccinate (30 min. reaction)	0.5	1.5	..	...
α-Ketoglutarate	7.5	1.0	..	...
Succinate	5.5	1.0	..	...
Fumarate	4.4	1.5	..	...
Malate	2.0	1.0	10.9	...
Oxalacetate	2.3	1.5	..	...
Pyruvate alone	...	0.2	..	...

carboxylic acid cycle is in plants and whether it is functional in mature plant tissues remain to be established.

Summary.—Methods for the chromatographic separation and determination of the 2,4-dinitrophenylhydrazones of pyruvate, α-ketoglutarate, oxalacetate, and oxalosuccinate are described. Recoveries greater than 98 per cent for 100 μg. quantities of pyruvate, α-ketoglutarate, and oxalacetate are obtained; the range for each acid is 10–500 μg.

Mitochondria isolated from the cotyledons of etiolated lupine seedlings oxidize pyruvate via the tricarboxylic acid cycle in a manner analogous to that of animal mitochondria. Individual experiments in which pyruvate-2-C^{14} plus unlabeled malate were oxidized by lupine mitochondria in the presence of one other acid of the tricarboxylic acid cycle demonstrated incorporation of C^{14} into each acid of the cycle so tested.

Acknowledgment.—We wish to thank Dr. C. Heidelberger for a tracer amount of pyruvamide-2-C^{14} used in the exploratory experiments and Dr. G. W. E. Plaut for a gift of DPN of high purity.

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*SUBCELLULAR FRACTIONATION OF MOUSE SPLEEN RADIATION PROTECTION ACTIVITY**

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Jacobson, *et al.*[1] have demonstrated that the survival of mice exposed to lethal doses of x-rays can be increased significantly by implantation of normal mouse spleens into the peritoneal cavity of irradiated mice after exposure. These findings have led to the concept of a humoral radiation recovery factor (or factors) present in, and elaborated by, normal mouse spleen tissue.[2] They were followed by experimental studies from this Laboratory,[3, 4] in which it was shown that the intraperitoneal injection of neutral phosphate buffer homogenates of normal mouse spleen into mice which had received otherwise lethal whole body x-irradiation, afforded marked protection against mortality. These studies provided a reproducible experimental procedure for obtaining active, injectable spleen preparations in which the vast majority of the cells had been disrupted, and in which the level of organization was far removed from that of the original spleen tissue. Furthermore, the experimental procedure provided a suitable biological assay for testing the protective activity of various spleen

fractions and for investigating the nature of the spleen factor. The supernatant obtained after centrifugation of the spleen homogenate at 1400 G for 10 minutes elicited no protective effect, while protective activity was retained in the sediment. In view of these results, it was considered plausible that the spleen factor—if a chemical entity—might be associated with, or bound to certain particulate or structural components comprising the spleen cells. In the present studies, normal mouse spleen tissue was subjected to subcellular fractionation by the Schneider and Hogeboom method of differential centrifugation in sucrose medium,[5, 6] and the respective subcell fractions assayed for radiation protection activity. The experimental data indicate that protective activity or mouse spleen homogenates is associated with the spleen *nuclei fraction*, under the conditions of these experiments.

Material and Methods.—The radiation procedure was essentially identical with that described previously.[4] A Westinghouse Therapy Unit was the x-ray source. Radiation factors were: 250 kvp.; 15 ma.; 0.5 mm. Cu plus 1 mm. Al filter; skin-to-target distance, 100 cm.; dose rate approximately 25 r per minute, measured in air with a Victoreen r-meter. In each experiment the control and experimental animals were matched with respect to sex, age, and body weight, irradiated simultaneously, and caged together, eight or ten per cage. The irradiated animals were LAf_1 mice of both sexes, 10 to 14 weeks old, weighing 20 to 25 g.; the spleen donors were non-irradiated young LAf_1 mice (approximately 1 week old).

Young (1-week-old) non-irradiated LAf_1 mice were sacrificed by cervical dislocation. The spleens were removed immediately (using clean but not aseptic technique), transferred to a tared chilled, sterilized Ten-Broeck glass homogenizer, weighed, and homogenized in 3 ml. of chilled 0.25 *M* sucrose solution. During this process the homogenizer was kept immersed in a water-bath containing cracked ice. The homogenate was diluted with 0.25 *M* sucrose solution to a final concentration of approximately 100 mg. spleen per ml. The diluted homogenate was then transferred to a chilled, graduated 15-ml. centrifuge tube, and centrifuged in a refrigerated International No. 2 centrifuge (5°C.) at 600 G for 10 minutes. The supernatant was withdrawn carefully with a pipette and transferred to a flask. The sediment was transferred to a clean homogenizer, rehomogenized in fresh 0.25 *M* sucrose solution, and made up to the original volume with 0.25 *M* sucrose solution. The homogenate was again centrifuged at 600 G for 10 minutes, the supernatant removed as before, and combined with that from the first centrifugation. The sediment from the second centrifugation, resuspended in 0.25 *M* sucrose solution, constitutes the *spleen nuclei* fraction. This fraction was turbid, whitish, and was easily injected through a No. 26 needle. The combined supernatants from the above

were then subjected to centrifugation in a refrigerated Model E Spinco preparatory ultracentrifuge at 8500 G for 10 minutes. The rotor was precooled to 5°C., and the vacuum chamber was refrigerated throughout the run. The supernatant fluid was carefully removed with a medicine dropper, and the sediment, constituting the *mitochondria fraction*, was resuspended in fresh sucrose solution. The supernatant from the mitochondria fraction was then centrifuged in the Spinco ultracentrifuge at 20,000 G for 90 minutes, yielding a sediment, the *microsome fraction*, which was resuspended in fresh 0.25 *M* sucrose. The supernatant from the microsome fraction was a pink, crystal-clear, optically homogeneous fluid. It constituted the *soluble fraction*. The individual subcellular fractions were administered without additional washing.

In the earlier experiments the medium used for subcellular fractionation was an aqueous solution of 0.25 *M* sucrose made up with distilled water. Since it was found that the protective activity of spleen homogenates prepared in this medium was not of a high order, it was decided to use a modified, sucrose medium containing added salts. The sucrose-salt solution had the following composition: 0.242 *M* sucrose, 0.0094 *M* KH_2PO_4, 0.0125 *M* K_2HPO_4, 0.0015 *M* $NaHCO_3$, and 0.0006 *M* adenosine triphosphate (Na ATP). This solution represents a modification of one used by Wilbur and Anderson[7] for isolating cell nuclei. It is isosmotic, fortified with potassium salts, bicarbonate, and adenosine triphosphate, and of suitable density to permit subcellular fractionation.

Thirty minutes after harvesting, the subcell fractions were injected either intraperitoneally or intravenously into the irradiated mice, usually between 2 and 3 hours after radiation exposure. Aliquots of each fraction injected never exceeded 1.0 ml., and the amount injected per mouse is expressed in terms of equivalent weight of spleen tissue from which it was derived. The control, irradiated mice, received injections of equivalent volumes of 0.25 *M* sucrose solution, or sucrose-salt solution. Survival up to 30 days post-irradiation and body weight changes were used as criteria for evaluation of protective activity in the spleen subcell fractions.

Both wet and stained dry preparations of whole spleen homogenate and of the particulate fractions were examined under the microscope. Cell counts were made using a standard hemacytometer.

Results.—It is apparent from examination of the data in table 1 that the 0.25 *M* sucrose solution was unsatisfactory as a medium for the preparation of active spleen homogenates. Whole spleen homogenates prepared in this medium exhibited some radiation protection activity, but to a far lesser degree than that reported previously for whole spleen homogenates in phosphate buffer at pH 7.2. Of the subcell fractions isolated in this medium, there was a suggestion of protective activity in the nuclei fraction, and to some extent also in the mitochondria. Whole spleen homog-

enates prepared in 0.88 *M* sucrose were completely ineffective in radiation protection.

In one experiment, spleen homogenate was prepared in a 0.25 *M* sucrose medium to which adenosine triphosphate (Na ATP, 5.7×10^{-4} *M*) had been added. Of ten mice, exposed to 750 r, each of which received a single intraperitoneal injection of this homogenate (equivalent to 56 mg. of spleen),

TABLE 1

THIRTY-DAY MORTALITY OF IRRADIATED MICE INJECTED WITH SUBCELLULAR SPLEEN FRACTIONS ISOLATED IN 0.25 *M* SUCROSE MEDIUM

RADIATION DOSE, R	FRACTION INJECTED	EQUIVALENT AMOUNT OF SPLEEN INJECTED, MG.	SURVIVAL AT 30 DAYS NO./TOTAL	SURVIVAL AT 30 DAYS PER CENT
700	Spleen homogenate in 0.25 *M* sucrose	48	6/10	60
700	Nuclei fraction	48	0/10	0
700	Supernatant from nuclei	70	0/10	0
700	0.25 *M* sucrose	..	0/10	0
750	Spleen homogenate in 0.25 *M* sucrose	80[a]	2/15	13
750	Nuclei fraction	80	2/15	13
750	Mitochondria fraction	80	1/15	7
750	Microsome fraction	80	0/6	0
750	Soluble supernatant fraction	72	0/6	0
750	0.25 *M* sucrose	..	0/15	0
750	Spleen homogenate in 0.25 *M* sucrose	42	2/13	15
750	Spleen homogenate in 0.88 *M* sucrose	42	0/13	0
750	0.25 *M* sucrose	..	0/6	0
750	0.88 *M* sucrose	..	0/6	0
750	Spleen homogenate in 0.25 *M* sucrose	42	1/10	10
750	Supernatant—70 G for 10 min.	42	0/12	0
750	Residue from above	42	2/12	17
750	0.25 *M* sucrose	..	0/12	0
750	Spleen homogenate in 0.25 *M* sucrose[b]	56	8/10	80
750	Nuclei fraction	280	3/9	33
750	Mitochondria fraction (intravenous)	250	0/5	0
750	Mitochondria fraction	220	2/7	29
750	0.25 *M* sucrose + ATP	..	0/10	0

[a] Donors, 2 weeks old.

[b] Contains 6×10^{-4} *M* adenosine triphosphate (ATP).

All injections were intraperitoneal, except as otherwise indicated.

eight were alive 30 days after radiation exposure, whereas none of the irradiated control mice receiving sucrose plus ATP survived. The spleen nuclei fraction, isolated in this medium, exhibited some protective activity: three out of nine survived of the irradiated mice each injected with the nuclei fraction (equivalent to 280 mg. spleen) the average survival time of the non-survivors was 15.5 days, compared to 9.2 days for the su-

crose controls. The mitochondria fraction when injected intraperitoneally (equivalent to 220 mg. spleen) also afforded a small degree of protection: two survivors out of seven; average survival time of non-survivors was 9.4 days. Intravenous injection of mitochondria did not afford protection against death; average survival time was 11.2 days.

TABLE 2

THIRTY-DAY MORTALITY OF IRRADIATED MICE INJECTED WITH SPLEEN SUBCELLULAR FRACTIONS ISOLATED IN SUCROSE-SALT MEDIUM

RADIATION DOSE, R	FRACTION INJECTED	EQUIVALENT AMOUNT OF SPLEEN INJECTED, MG.	SURVIVAL AT 30 DAYS NO./TOTAL	SURVIVAL AT 30 DAYS PER CENT
750	Spleen homogenate in sucrose-salt	56	7/17	41
750	Nuclei fraction	280	9/9	100
750	Mitochondria fraction	250	0/8	0
750	Microsomes + soluble supernatant	140	0/8	0
750	Sucrose-salt solution	..	0/10	0
750	Spleen homogenate in sucrose-salt	56	10/10	100
750	Nuclei fraction	280	10/10	100
750	Mitochondria (intravenous)	250	0/8	0
750	Nuclei fraction	196	5/7	71
750	Nuclei fraction	98	2/3	67
750	Sucrose-salt solution	..	0/8	0
750	Spleen homogenate in sucrose-salt	39	5/5	100
750	Nuclei fraction	168	3/8	38
750	Nuclei fraction in 1 *M* NaCl	150	0/6	0
750	Controls (no injection)	..	0/11	0
750	Spleen homogenate in sucrose-salt	56	7/8	87
750	Nuclei fraction	196	2/4	50
750	Salt-sucrose solution	..	0/7	0
750	Spleen homogenate in sucrose-salt	56	3/5	60
750	Nuclei fraction in 0.5 *M* NaCl	280	0/4	0
750	Salt-sucrose solution	..	0/5	0
750	0.5 *M* NaCl solution	..	0/5	0
750	Nuclei fraction in 1 *M* NaCl, diluted to 0.75 *M* NaCl	250	0/6	0
750	0.75 *M* NaCl solution	..	0/10	0

All injections were intraperitoneal, except as otherwise indicated.

Subcell Fractionation in Sucrose-Salt-ATP Medium.—The results indicate that the modified sucrose-salt-ATP solution provided an excellent medium for the preparation of protective spleen homogenates, which could then be subjected to subcellular fractionation. The survival data are summarized in table 2. The degree of post-irradiation protection afforded by the injection of spleen homogenates prepared in the sucrose-salt medium was greater than that observed with comparable homogenates prepared in

0.25 *M* sucrose medium. This is also illustrated by comparative body weight changes of mice exposed to 750 r and treated with spleen homogenates prepared in both media. (See figure 1.)

In a series of five separate experiments, marked protection against radiation mortality was afforded by a single injection of the spleen nuclei fraction. In two experiments 100 per cent of the irradiated mice (750 r), each injected with nuclei fraction from ten spleens, survived the 30-day observation period, whereas all of the irradiated controls (750 r) injected with the sucrose-salt solution died. Apparently, the amount of spleen substance in the form of the nuclei fraction required for protective activity

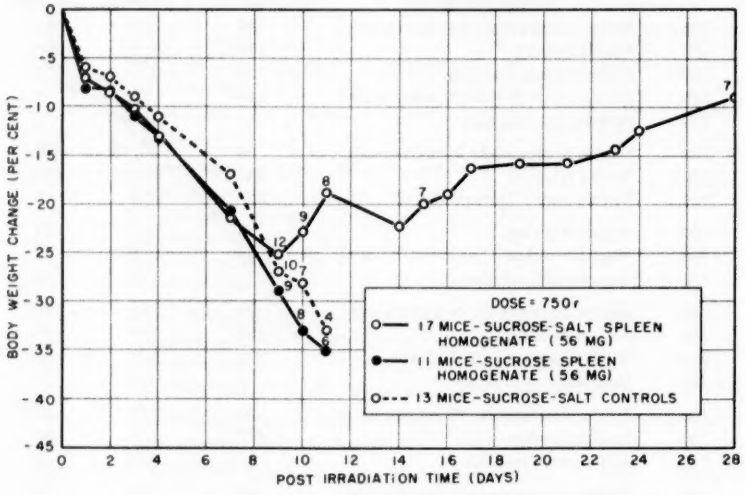

FIGURE 1

Body weight changes in x-irradiated mice treated with spleen homogenates prepared in sucrose-salt medium and in 0.25 *M* sucrose

is greater than that in the form of whole spleen homogenate. However, some activity was observed with the nuclei fraction derived from as little as 3.5 spleens (98 mg.) was administered to irradiated mice.

No evidence of radiation protection activity was observed in the mitochondria fraction obtained by differential centrifugation in the sucrose-salt medium (table 2), in contrast with a small increase in the number of survivors in the group of irradiated mice injected with the mitochondria fraction isolated in 0.25 *M* sucrose medium. Since it is known that mitochondria become aggregated and agglutinated in the presence of salts, and as a result are sedimented with the nuclei,[9] it seems likely that the nuclei fraction isolated in the sucrose-salt medium contains a considerable

admixture of aggregated mitochondria. These considerations suggest that a small degree of radiation protection activity may well be associated with the mitochondria fraction, but that the apparent lack of activity in the mitochondria isolated in sucrose-salt medium may be ascribable to the smaller amount of mitochondria injected.

Desoxyribonucleic acids, in association or combination with protein, comprise the major portion of the cell nucleus. Furthermore, Schneider[10] has shown that all of the desoxyribonucleic acid in liver, kidney, and tumors is recovered in the nuclei fraction. Desoxyribonucleic acid constitutes as much as 48.5 per cent by weight of the dry, fat-free salmon sperm nuclei[11] and indeed, among the various tissues of the animal body, the spleen is one of the richest in desoxyribonucleic acid content.[12] On the basis of the present finding that the radiation protection activity of mouse spleen homogenates is associated with the cell nuclei fraction, an attempt was made to determine whether crude nucleic acid extracts from the nuclei fraction would exhibit radiation protection activity. The procedure of Mirsky and Pollister[13] involving the extraction of nucleoprotein by 1 *M* NaCl was employed. The spleen nuclei fraction was first prepared in sucrose-salt medium as described above; one-half of the nuclei suspension was injected into a group of eight mice approximately 1 hour after exposure to 750 r. Solid NaCl, to a final concentration of 1 *M* NaCl, was added to the remaining half of the spleen nuclei suspension in a flask immersed in an ice-water bath. Immediately upon addition of the salt, the nuclei suspension became very viscous and quite resistant to flow. Since it could not be readily subjected to centrifugation, the viscous mass was administered directly to a second group of irradiated mice (750 r). The material was administered by intraperitoneal injection through a No. 23 needle, after packing it into the barrel of the hypodermic syringe with a glass rod. The data in table 2 show that the viscous 1 *M* NaCl suspension of the nuclei fraction was devoid of radiation protection activity, as was the spleen nuclei fraction suspended in 0.5 *M* NaCl solution.

Inactivation of Spleen Homogenates by in Vitro Exposure to X-Rays and to Ultrasonic Vibrations.—Additional experimental data pertinent to the present discussion show the effect of x-radiation, and of ultrasonic vibrations on the radiation protection activity of mouse spleen homogenates. The homogenate was prepared and divided into two equal portions. One portion was exposed to the physical agent while the other portion, although kept under otherwise identical conditions, was not exposed. The treated and untreated homogenates were then respectively injected into two groups of irradiated animals. The source of the ultrasonic radiations was a crystal Ultra-Sonorator (Crystal Research Laboratories). The spleen homogenate, contained in a Pyrex test-tube, was immersed in the oil bath of the Ultra-Sonorator during the exposure. In one experiment, the

homogenate was exposed continuously for 5 minutes at 1010 v. and 160 ma. In the second experiment, the ice-chilled homogenate was exposed at 750 v. and 100 ma. for a total time of 4 minutes; the exposure was alternated 1 minute in the Ultra-Sonorator and 1 minute in an ice-water bath. It seems clear from the data presented in table 3 that both x-ray exposure (750 r), and ultrasonic vibrations, in vitro, inactivate the protective activity of mouse spleen homogenates. Relative body weight changes in x-irradiated mice treated with normal spleen homogenate, and with spleen homogenate exposed to 750 r are shown in figure 2.

Microscopic Observations.—An attempt was made to ascertain the number of whole cells, broken cells, and cell nuclei in whole spleen homogenates and in nuclei fraction. The attainment of this objective is far more difficult than would appear at the outset, since adequate microscopic criteria for distinguishing intact spleen cells from partially disrupted ones and from nuclei, are not readily established. In order to obtain base line criteria for the normal appearance and size of intact spleen cells, imprints of young mouse spleens were prepared. The freshly cut surface of the spleen was touched lightly on a glass slide; the slide was then air-dried and stained with Wright-Giemsa stain. Lymphocytes of all sizes were seen, as well as reticulum cells, megakaryocytes, normoblasts, polymorphonuclear leukocytes, blast cells, and an occasional eosinophil. The vast majority of the cells ranged in diameter from 6 μ to 13 μ. A few macrophages 19–20 μ in diameter were seen. The cell nuclei stained dark purple and comprised the largest proportion of the cell diameter. The cytoplasm appeared as pale blue rims around the nuclei.

TABLE 3

INACTIVATION OF SPLEEN HOMOGENATE RADIATION PROTECTION ACTIVITY BY IN VITRO EXPOSURE TO X-RAYS, OR TO ULTRASONIC VIBRATIONS

RADIATION DOSE R	MATERIAL INJECTED	SURVIVAL AT 30 DAYS NO./TOTAL	SURVIVAL AT 30 DAYS PER CENT
650	Spleen homogenate (32 mg.)[a]	10/10	100
650	Spleen homogenate (32 mg.) exposed to ultrasonic vibrations	8/17	47
650	Phosphate buffer	8/13	62
700	Spleen homogenate (36 mg.)	14/15	93
700	Spleen homogenate (36 mg.) exposed to ultrasonic vibrations	3/15	20
700	Phosphate buffer	1/10	10
750	Spleen homogenate (56 mg.)	7/10	70
750	Spleen homogenate (56 mg.) exposed to 750 r	0/10	0
750	Phosphate buffer	0/10	0

[a] Equivalent amount of spleen.

Microscopic examination of the stained preparations of phosphate buffer spleen homogenate revealed that the vast majority of cells in this preparation are obviously disintegrated or disrupted. A few apparently intact cells resembling small lymphocytes were seen; the remainder of the preparation consisted of a great number of deeply staining, free nuclei, a number of partially disintegrated cells in which portions of the cell surface had been torn away, and granular cell debris. Cell counts indicated the presence of approximately 4.3×10^7 cellular bodies, i.e., whole cells plus disrupted cells plus nuclei, in sucrose-salt homogenates derived from 100 mg. of spleen. Of these bodies, approximately 3.6×10^6 appeared to be

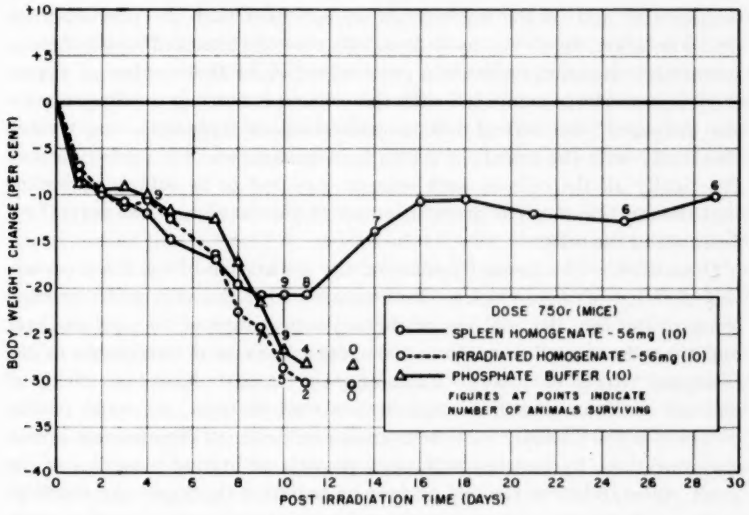

FIGURE 2

Effect of in vitro exposure of spleen homogenates to x-rays (750 r) on radiation protection activity

intact cells. On the basis of the value of 22.9×10^6 cells per milligram of normal mouse spleen recently reported by Mizen and Petermann,[14] it was calculated that the single homogenization involved in the preparation of whole spleen homogenates had disrupted approximately 80 per cent of the spleen cells beyond recognition as cells or cell bodies. Of the remaining 20 per cent recognizable as cell bodies, approximately one-fifth appeared to be whole cells. Thus, the spleen homogenate contains about 2 per cent of the original number of cells in seemingly whole form. It will be recalled that the whole homogenate derived from one mouse spleen will afford marked protection against radiation mortality. Assuming that the

remaining whole cells after homogenization are representative of the spleen population, implantation of one-fiftieth of one spleen would almost certainly not provide the same order of radiation protection (if any) as that afforded by spleen homogenate derived from one spleen and containing the same number of whole cells as that contained in one-fiftieth of one spleen. The additional protection, then, must have come from the disrupted cells.

Microscopically, the nuclei fraction consisted largely of nuclei which were, in many instances, disrupted. The number of seemingly whole cells in this fraction was very low, and indeed, many microscopic fields were entirely devoid of whole cells. Visual comparison of the smear of the whole homogenate and of the nuclei fraction indicates that the procedure for nuclei isolation, involving, as it does, two separate homogenization steps in sucrose-salt medium, results in a great reduction of the number of apparently intact cells as compared with the original homogenate. Smears were also prepared from normal unhomogenized spleen fragments, and treated identically with the smears of spleen homogenates and the nuclei fraction. Practically all the cells in such smears appeared to be intact, indicating that the method of smear preparation *per se* did not disrupt the normal appearance of the cells.

Discussion.—The present finding of the association of radiation protection activity of mouse spleen homogenates with the cell nuclei fraction bring to the fore the problem of the radiosensitivity of the cell nucleus, and the role of nucleic acids and nucleoproteins as determinants in the biological effects of x-rays. Certainly, the earliest damaging effects of ionizing radiations, such as inhibition of cell division, are most readily observed in the nucleus. The well-known induction of chromosome breaks and mutations by ionizing radiations provide additional examples of the great vulnerability of the cell nucleus to radiation damage. An excellent review of the problem of radiation sensitivity of the nucleus has been written by Sparrow.[15]

The experimental evidence is available to support the supposition that the primary disturbance—on the biochemical level—elicited by ionizing radiations may be an aberration in the chain of metabolic events leading to the biosynthesis or utilization of nucleoproteins. Thus, Hevesy[16] observed that x-irradiation inhibits the incorporation of C^{14} carboxy-labeled acetate into desoxyribonucleic acid (DNA) purines. He stated that the finding "that the rate of formation of a main constitutent of cell nuclei is reduced appreciably after irradiation helps to explain the blocking effect of irradiation on cell division and the formation of anomalous mitotic products in the irradiated tissue." Skipper and Mitchell[17] demonstrated a similar inhibition of incorporation of radioactive (C^{14}) bicarbonate and formate into viscera nucleic acids and nucleic acid purines and mice, 6 hours after ex-

posure to x-rays (950 r). Furthermore, the work of Abrams[18] and of Holmes[19] shows that the rates of biosynthesis of nucleic acids are reduced markedly by x-rays, *even though the synthesis of proteins is relatively unaffected.* Also pertinent to this discussion is the observation of x-ray-induced depolymerization of thymonucleohistone and of Na thymonucleate.[20, 21] Limperos and Mosher[22] attempted to demonstrate this effect in vivo. Rats receiving 1000 r whole-body irradiation, were sacrificed either immediately or 24 hours after exposure, and the thymus DNA was isolated and analyzed. The results indicated that a splitting off of either purine or pyrimidine bases had occurred in the DNA isolated from the rats immediately sacrificed; the thymus DNA isolated from animals sacrificed 24 hours after irradiation was almost completely depolymerized, as revealed by the structural viscosity, streaming birefringence, and sedimentation constants of the DNA solutions. Also of considerable significance to the present discussion is the experimental demonstration of nucleic acid depolymerization by chemically produced hydroxyl radicals,[23] and the evidence for an oxygen effect in the action of x-radiation on nucleic acid in vitro.[24]

The present finding of radiation protection activity in the spleen nuclei fraction and the destruction of this activity by treatment of the nuclei fraction with 1 *M* NaCl, or by x-irradiation of spleen homogenate in vitro appears to be in line with above observations when viewed in the light of the following hypothesis: It is postulated (*a*) that the cell nuclei of normal mouse spleen contain a specific macromolecular nucleoprotein complex which is required for the cell growth and division, regeneration, or maturation of critical hematopoietic tissue (bone marrow, spleen); (*b*) that the biological activity of this nucleoprotein is inhibited by ionizing radiations. The inhibition may result from interference with the biosynthesis of this compound, or as a consequence of a physico-chemical alteration of the nucleic acid moiety which may result in depolymerization. According to the above hypothesis, the exposure of mice to an LD_{100} dose of total body x-irradiation, would result in a cessation or inhibition of the new-formation of the specific nucleoprotein complex. A deficiency in the supply or biological activity of this complex would then lead to a defect in the production or new-formation of leukocytes and erythrocytes and, since the biological half-life of the circulating leukocytes is known to be much shorter than that of the erythrocytes, the defect in hematopoiesis at the site of the formation would be manifested in the characteristic leukopenia of the acute radiation syndrome—the essential features of which can indeed be largely explained on the basis of cell damage leading to deficiencies that are first apparent in areas of rapid cell turnover, e.g., hematopoietic and intestinal epithelial tissues.

The biological activity of the postulated nucleoprotein may, therefore,

be the limiting factor in the recovery of mice exposed to LD_{100} total body x-rays; and the administration of the spleen nuclei fraction (obtained from normal, non-irradiated mice) to irradiated mice would provide a new source of the intact, non-degraded, biologically active nucleoprotein complex, able to stimulate or elicit hematopoietic recovery in the irradiated host mouse, and thus prevent death.

The present observations that exposure of spleen homogenates to x-radiation (750 r) and to ultrasonic vibrations, in vitro, results in loss of radiation protection activity are consistent with the above hypothesis, since both these physical measures are known to induce degradation and depolymerization of nucleic acid macromolecules.[25]

In view of the functional similarities betweeen the spleen and bone marrow in the mouse,[26] and the fact that injection of mouse bone marrow suspensions into irradiated mice affords them marked protection against death,[27] it seems likely that the protective factors in spleen and bone marrow are closely related in nature, or even identical. This conclusion finds some support in the observation that the sensitivity of splenectomized mice to total body x-irradiation does not differ appreciably from that of intact, non-splenectomized mice.[28] Presumably the spleen and bone marrow factors can replace each other. It is significant, too, that spleen and bone marrow, both tissues of high cell turnover, appear to be the major sources of the radiation protection factor in mice. In this connection it is of interest that Kelly and Jones[29] were able to demonstrate an alteration in the turnover of desoxypentose nucleic acids in the liver, spleen and kidneys of mice bearing growing mammary carcinoma transplants. These workers suggested "that the presence of a rapidly dividing tissue mass may influence the rate of cell division (as evidenced by the nucleic acid turnover rate) in the other body tissues."

Finally, it seems pertinent to note at this point that thiourea inhibits the depolymerization of DNA by x-radiation and by chemically generated free radicals, whereas ascorbic acid (another reducing agent) does not have this action.[23] Since thiourea protects rats against lethal x-ray doses when given prior to irradiation[30] while ascorbic acid administration confers no radiation protection,[31] it would appear plausible that preirradiation protection by compounds such as thiourea or cysteine may be related to their ability to inhibit DNA depolymerization by x-rays. Thus, radiation protection by these compounds would involve "protection" of the postulated spleen nucleoprotein factor against depolymerization and resultant loss of biological activity. Cronkite, *et al.*,[26] have previously suggested that the accelerated recovery of the bone marrow and of splenic myelopoiesis in glutathione-pretreated irradiated mice "may be due to protection of humoral factors which control orderly hemopoietic regeneration."

The experimental data presented in this paper strongly suggest that the

post-irradiation protection factor present in the spleens of young LAf_1 mice is non-cellular in nature and is associated with the cell nuclei. Studies aimed at the isolation and further identification of the factor are now in progress.

Summary.—The subcellular particulate fractions of mouse spleen were isolated by differential centrifugation of spleen homogenates in sucrose media. The experimental data indicate that post-irradiation protection activity of mouse spleen homogenates is associated with the cell nuclei fraction. The highest degree of protective activity was exhibited by the nuclei fraction isolated in a modified, isosmotic sucrose-salt medium fortified with adenosine triphosphate. In two of the experiments 100 per cent of the irradiated mice (750 r), each injected with the nuclei fraction derived from 280 mg of spleen tissue, were alive 30 days after exposure, whereas all of the irradiated controls (750 r) died. No activity was observed in the mitochondria, microsome, or soluble supernatant fractions isolated in this medium. A low level of protective activity was observed in whole spleen homogenates and in the spleen nuclei fraction prepared in 0.25 *M* sucrose medium. Crude nucleic acid extracts prepared by treating the nuclei fraction with 1 *M* NaCl were inactive. The data strongly suggest that the radiation protection spleen factor is non-cellular in nature. It is proposed that the factor may be a nucleoprotein.

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THE MECHANISM OF PROPIONIC ACID FORMATION BY SUCCINATE DECARBOXYLATION. I. THE ACTIVATION OF SUCCINATE

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The studies of Johns[1–3] and Delwiche[4] with suspensions of anaerobic micrococci (*Micrococcus lactilyticus* or *Veillonella gazogenes*) and *Propionibacterium* spp. showed that these bacteria produced propionate by the decarboxylation of succinate. This decarboxylation can also be accomplished by cell-free extracts of *M. lactilyticus* and the requirement for specific cofactors in this reaction has been briefly described.[5] The present paper contains detailed evidence for the participation of these cofactors in the activation of succinate and for the nature of the active product.

Methods.—*M. lactilyticus* (strain 221) was grown in tap water with 2–3% sodium lactate, 2% peptone, 1% yeast extract, salts, and 0.0015% thiamin. The latter was added routinely to all media, except when otherwise noted, because some batches of yeast extract, apparently deficient in thiamine, yielded small cell crops and extracts of such cells had low decar-

boxylase activity on a unit protein basis. When the deficient media were supplemented with thiamin, greater cell crops resulted, and extracts prepared from these cells had a high activity.

Cultures were incubated at 37°C. for 16–24 hours; the cells were then harvested, washed once in tap water, ground with alumina,[6] the cell paste extracted with distilled water, or with a 0.01% solution of a reducing agent, and centrifuged at approximately 12,000 × g for 20 minutes. Extracts so prepared, with cysteine as the reducing agent, could be stored for several days in the frozen state without great loss in activity.

Details of manometric procedure and of methods for the determination of volatile acids have been published elsewhere.[7] The protein content of the extracts was estimated by the method of Stadtman, *et al.*[8]

Total hydroxamic acid was determined according to Lipmann and Tuttle;[9] identification and quantitative estimation of the component acids was accomplished by paper chromatography.[10] For the separation of succinhydroxamic acid from other hydroxamic acids a 10:2 mixture of phenol-water was used.

TABLE 1

THE EFFECT OF pH ON THE DECARBOXYLATION OF SUCCINATE

pH	μL. CO_2/10 MG. EXTRACT PROTEIN/HOUR
5.0	490
5.3	543
5.5	560
5.7	520
6.0	512
6.5	359
7.0	199

Pabst Co. preparations of adenosine-tri-phosphate (ATP) and coenzyme A(CoA, approximately 200 units/mg.) were used. Concentrations of CoA are expressed in terms of units as defined by Kaplan and Lipmann.[11]

CoA and ATP were adsorbed from the extracts by treatment with Dowex-1 and Norit A;[8] 98% of the CoA, and 90–95% of the ATP content were thus removed. The former value was established by CoA assay with pigeon-liver enzyme,[11] the latter by measurement of absorption at 260 mμ.

Dialysis was performed at 5°C. against a 0.001% cysteine solution at pH 5.5; if continued too long, the extracts are permanently inactivated. Consequently the optimal duration of dialysis was established by determining the activation of the extracts at frequent intervals.

Results.—Conditions for testing the decarboxylation of succinate: All experiments were conducted under anaerobic conditions inasmuch as in air succinate is not decarboxylated but oxidized to acetate. The optimum

substrate concentration for carbon dioxide production is approximately 20 μM/ml. with 20 mg. of extract protein. The highest rate of decarboxylation occurred at pH 5.5 (table 1), and all experiments in which carbon dioxide production was measured were performed at this pH. With freshly prepared extracts the rate of decarboxylation was approximately the same in various buffer solutions (acetate, propionate, benzoate, man-

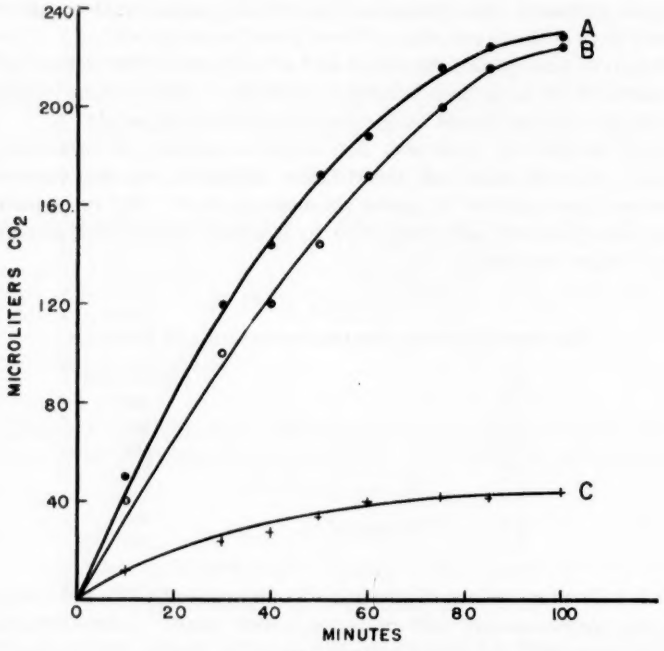

FIGURE 1

Decarboxylation of succinate by extracts of *M. lactilyticus*. A: Original extract, 10 μM succinate. B: Supernatant fluid after centrifugation at 60,000 $\times$ g, 10 μM succinate. C: Sediment after centrifugation at 60,000 $\times$ g, 10 μM succinate; also endogenous, original extract.

delate, and phthalate); citrate buffer was strongly inhibitory, and phosphate caused a slight decrease in the rate of decarboxylation. Since $MgCl_2$ partially relieved the inhibition by citrate, but not by phosphate, the action of the former was attributed to the binding of metal ions. The addition of $MgCl_2$ and reducing agents increased the rate of succinate decarboxylation, the latter probably by maintaining the cofactors in the reduced (active) state. Cysteine, glutathione, thioglycollate, and 1,3-

dimercaptopropanol at a final concentration of approximately 0.01 *M* were equally effective; Na_2S was toxic. NaF was added to inhibit an ATP-ase present in the crude extracts. Thus, carbon dioxide production from succinate by freshly prepared bacterial extracts was measured at 30°C., in N_2, with the following reaction mixture: 1 ml. extract containing 10–20 mg. protein, 10 μM reducing agent, 10 μM $MgCl_2$, 100 μM NaF, 50 μM succinate, and 50μM buffer, the choice of buffer depending on the nature of the experiment; total volume 2.2 ml.

Figure 1 shows the production of carbon dioxide from succinate by an extract of *M. lactilyticus*. If an extract prepared as described is further centrifuged at approximately 60,000 × g for 40 minutes, a small amount of sediment is found. This sediment is not capable of decarboxylating succinate, but the supernatant fluid is, and at a rate comparable to that determined for the original extract. This shows that the enzymes involved in the decarboxylation of succinate are soluble.

Suspensions of intact cells decompose succinate to equimolar amounts of carbon dioxide and propionate;[1, 2] this is true also for extracts. The decomposition of succinate is therefore a straight decarboxylation.†

Cofactor requirements: Freshly prepared extracts, tested under the above conditions, decarboxylate succinate rapidly without the addition of any cofactors (Fig. 1). As described previously,[5] an increase in the rate of carbon dioxide production occasionally results from the addition of cocarboxylase. This effect is restricted to extracts prepared from cells grown in a thiamine-deficient medium, or to extracts subjected to prolonged dialysis.

Experiments with cell suspensions of biotin-deficient *Propionibacterium pentosaceum*[12] showed that biotin increased the rate of succinate decarboxylation. Since *M. lactilyticus* does not require this cofactor for growth,[13] biotin-deficient cells could not be obtained. However, the addition of biotin to untreated or "aged" cell suspensions,[14] or to freshly prepared, dialyzed, or depleted extracts did not increase the rate of succinate decarboxylation. Adenylic acid and a factor prepared by autoclaving glucose with H_2SO_4 have been reported to be involved in biotin-mediated reactions;[15, 16] their addition, singly or in combination with biotin, did not affect the decarboxylation of succinate by the above-described preparations of *M. lactilyticus*.

Freshly prepared extracts respond to the addition of ATP and CoA, although a greater increase in decarboxylase activity upon the addition of these cofactors can be demonstrated with extracts that have been dialyzed or stored in the frozen state. The greatest effect is observed with preparations from which CoA and ATP have first been removed by treatment with adsorbents. Such extracts have low activity, but almost complete reactivation can be accomplished by adding both ATP and CoA, in the

presence of cocarboxylase,[5] provided the pretreatment of the extract has not resulted in significant inactivation of the enzymes concerned.

If traces of ATP remain in the extract after adsorption, the addition of CoA alone is sufficient to cause considerable carbon dioxide production.[5] However, a more complete removal yields extracts that do not respond to the addition of either cofactor alone; both ATP and CoA are required for succinate decarboxylation. The rate of carbon dioxide production by such extracts increases, though not proportionally, with increasing amounts of ATP. The experimental results, summarized in table 2, show that the addition of even small amounts of ATP causes the decomposition of large amounts of succinate in the presence of CoA.

TABLE 2

THE EFFECT OF ATP AND CoA ON THE DECARBOXYLATION OF SUCCINATE BY DEPLETED EXTRACTS

	NO CoA ADDED		18 UNITS CoA ADDED				
μM ATP added	0	10.0	0	1.0	2.5	5.0	10.0
μl. CO_2/10 mg. extract protein/hour	46	40	56	226	234	338	380

TABLE 3

THE EFFECT OF ATP ON THE FORMATION OF SUCCINHYDROXAMIC ACID BY A DEPLETED EXTRACT

μM ATP ADDED	μM HYDROXAMIC ACID/10 MG. EXTRACT PROTEIN/HOUR
0	0.2
5.0	1.7
10.0	3.5
15.0	4.0
20.0	4.2
10.0	3.1[a]
10.0	0.0[b]

Reaction mixture contained: 1000 μM hydroxylamine, 500 μM TRIS buffer pH 7.3, 50 μM succinate, 10 μM glutathione, 10 μM $MgCl_2$, 100 μM NaF; incubated at 30°C. under pure N_2.

[a] Hydroxylamine added at end of incubation period.

[b] Reaction mixture heated for 5 minutes at 100°C. (pH 5.0) at end of incubation period and hydroxylamine added.

Activation of succinate: The reactivation of depleted extracts by ATP and CoA indicates an initial conversion of succinate to succinyl-CoA or succinyl phosphate prior to decarboxylation. This inference is supported by the fact that a hydroxamic acid, identified as succinhydroxamic acid by chromatography, is formed in extracts to which hydroxylamine has been added. The following observations suggest that, under certain conditions, the substance reacting with hydroxylamine is succinyl phosphate rather than succinyl-CoA.

The addition of hydroxylamine at the start of the incubation period completely suppresses carbon dioxide evolution; the amount of succinhydroxamic acid formed under these conditions does not depend on the quantity of CoA present,[5] but is solely determined by the amount of ATP (table 3). The addition of hydroxylamine at the end yields only traces of hydroxamic acid if sufficient CoA is present; in this case, decarboxylation of succinate occurs. With extracts supplied with ATP, but unable to decarboxylate succinate owing to the virtually complete removal of CoA, nearly the same amount of succinhydroxamic acid is obtained when hydroxylamine is added at the end, as when it was present from the start. Since the quantity of activated succinate produced in the absence of hydroxylamine by depleted

TABLE 4

THE EFFECT OF VARIOUS SUBSTANCES ON CARBON DIOXIDE PRODUCTION AND SUCCINHYDROXAMIC ACID FORMATION

ADDITION[a]	IN % OF CONTROL	
	CO_2	HYDROXAMIC ACID
Potassium arsenate	5	1
Sodium chloride	32	63
Lithium chloride	57	70
Potassium phosphate	20	130

[a] Final concentration 0.01 *M*.

TABLE 5

THE EFFECT OF ARSENATE ON THE FORMATION OF SUCCINHYDROXAMIC ACID BY A DEPLETED EXTRACT

UNITS CoA ADDED	μM ARSENATE ADDED			
	0.0	20.0	50.0	100.0
0	1.53	1.56	1.43	1.33
9	..	0.28	1.20	0.00
18	..	0.10	0.00	..
36	1.50[a]	0.05	..	..

Values given as μM hydroxamic acid/10 mg. extract protein/hour; 10 μM ATP in all reaction mixtures; experimental conditions listed under table 3; hydroxylamine added at end of incubation period.

[a] Hydroxylamine added at start of incubation period.

extracts depends on the ATP concentration, and far exceeds the trace amounts of CoA present, it appears likely that succinyl phosphate rather than succinyl-CoA is the compound giving rise to succinhydroxamic acid.

If the reaction mixture resulting from the incubation of a depleted extract, succinate, and ATP is heated for 5 minutes at 100°C. (pH 5.0), the addition of hydroxylamine no longer yields hydroxamic acid (table 3). Hence, the precursor of the latter is heat labile, a characteristic of acyl-phosphates, but not of acyl-CoA compounds,[17] although Sanadi and Littlefield[18] recently found that CoA esters may also be heat labile in crude preparations. However, in an aliquot of the *M. lactilyticus* extract used for testing the stability of the hydroxamic acid precursor, acetyl-CoA

was not decomposed by heat, thus supporting the proposal that the former is succinyl phosphate, rather than succinyl-CoA.

Supplementary evidence of a more indirect nature for the formation of a phosphate derivative comes from experiments with arsenate. The addition of arsenate completely inhibits carbon dioxide evolution and the production of succinhydroxamic acid (table 4), provided CoA is present. With increasing amounts of CoA, the arsenate inhibition of succinhydroxamic acid formation also increases (table 5). This effect can be accounted for by assuming a reversible, enzyme-mediated, reaction between succinyl phosphate and CoA with the formation of phosphate and succinyl-CoA. Substitution of arsenate for phosphate in the back reaction yields CoA and succinyl arsenate, followed by an instantaneous hydrolysis of the latter. This is analogous to the mechanism proposed for the disappearance of acetyl phosphate in solutions with phosphotransacetylase, arsenate, and CoA.[19]

Experiments on the effect of Na^+, Li^+, and $PO^{\equiv}_4$, on the activity of extracts also support the view that succinyl phosphate is produced (table 4). These ions differentially inhibit carbon dioxide production and the formation of succinhydroxamic acid. Na^+ and Li^+ inhibit both, but the addition of $PO^{\equiv}_4$ causes an increase in succinhydroxamic acid production and a marked decrease in carbon dioxide evolution. It is known that Na^+ and Li^+ have an inhibitory effect on phosphotransacetylase;[19] the action of $PO^{\equiv}_4$ is explicable by postulating the reversal of a reaction in which phosphate is an end-product, such as the above-mentioned reaction between succinyl phosphate and CoA.

The results of these experiments therefore show that succinate is initially activated in cell-free extracts of *M. lactilyticus*. The ATP-dependent accumulation of a substance capable of forming succinhydroxamic acid suggests that under certain conditions succinyl phosphate is the "active" form of succinate; experiments with inhibitors imply that succinyl-CoA may be formed. Evidence has recently been obtained for the formation of the both compounds by heart muscle preparations during the oxidation of alpha ketoglutarate.[20, 21]

Succinyl phosphate may be produced in the bacterial extract either from succinate and ATP, or by the initial formation of succinyl-CoA from succinate, ATP and CoA, followed by trans-succinylation.

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† The production of equivalent amounts of carbon dioxide and propionate from

succinate can be explained most simply as the result of succinate decarboxylation and this interpretation is used in the present papers. It is, however, recognized that experiments with purified enzymes may show that the process involves successive oxidations of four-carbon compounds and reductions of three-carbon compounds.

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THE MECHANISM OF PROPIONIC ACID FORMATION BY SUCCINATE DECARBOXYLATION. II. THE FORMATION AND DECARBOXYLATION OF SUCCINYL-CoA

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The effects of arsenate, phosphate, Na^+, and Li^+, described in the preceding paper,[1] suggest that succinyl-CoA is enzymatically produced from succinyl phosphate and CoA in extracts of *Micrococcus lactilyticus*. The CoA requirement for succinate decarboxylation thus indicated that succinyl-CoA might be the actual substrate for the decarboxylase. Evidence is presented here for the decarboxylation of succinyl-CoA and the mechanism of its regeneration in such extracts.

Methods.—Sulfanilamide acylation was tested by the method of Kaplan and Lipmann,[2] sulfanilamide being determined according to Bratton and Marshall.[3]

Pyrophosphate formation was investigated under the conditions described by Lipmann, *et al.*;[4] pyrophosphate was estimated by acid hydrolysis,[5] and enzymatically with yeast pyrophosphates,[6] orthophosphate by the method of Sumner.[7] Pyrophosphatase-free extracts were obtained by treatment of the crude extracts with the calcium phosphate gel which does not absorb pyrophosphates,[8] and elution of the gel with buffer at pH 5.7.

Succinoxidase was purified by the method of Keilin and Hartree.[9] Acetyl phosphate was synthesized from isopropenyl acetate and phosphoric acid;[10] succinyl-CoA and propionyl-CoA, according to Simon and Shemin.[11]

Results.—Decarboxylation of succinyl-CoA: Extracts depleted of ATP and CoA by treatments with adsorbents are unable to decarboxylate succinate.[1] However, the addition of succinyl-CoA to such extracts results in a rapid evolution of carbon dioxide (table 1, exp. 1). The fact that succinyl-CoA decarboxylation proceeds at a much greater rate than that of succinate in the presence of free CoA precludes the possibility of attributing the results to a preliminary decomposition of succinyl-CoA by a deacylase.

TABLE 1

CARBON DIOXIDE PRODUCTION BY DEPLETED EXTRACTS FROM SUCCINYL-CoA AND SUCCINATE WITH VARIOUS ADDITIONS

ADDITIONS	EXP. 1 10 μM SUCCINATE	EXP. 2 50 μM SUCCINATE	EXP. 3 50 μM SUCCINATE
None	36	61	20
10 μM ATP	35	70	22
18 units CoA	58	87	38
360 units CoA[a]	82	110	..
10 μM ATP, 18 units CoA	143	180	141
10 μM succinyl-CoA[c]	232[b]	..	..
1 μM propionyl-CoA	..	244	..
4 μM acetyl phosphate	..	..	19
4 μM acetyl phosphate, 18 units CoA	..	..	181

Values given in μl/10 mg. extract protein/hour.

[a] Amount of CoA used in preparing propionyl-CoA.

[b] Succinate omitted.

[c] Bicarbonate-free preparation.

The decarboxylation of succinyl-CoA may be represented either as a process yielding directly carbon dioxide, propionate, and free CoA, or as a reaction in which one of the end-products is released as a CoA compound. The latter possibility was examined by incubating succinate, ATP, CoA, and an amount of hydroxylamine insufficient to cause a complete inhibition of succinate decarboxylation, with an extract capable of activating succinate but virtually incapable of activating free propionate. Chromatography of the reaction mixture disclosed the presence of both succin- and propionhydroxamic acids, and comparison of the quantity of the latter

formed from succinate with that produced in a similar reaction mixture containing free propionate instead of succinate showed that the amount produced from succinate was by far the greater (table 2). Of special significance is the close correspondence between the quantities of propionhydroxamic acid recovered and carbon dioxide liberated during the decomposition of succinate. Under the experimental conditions used, the action of CoA transphorase, described below, cannot be detected. These results strongly suggest that propionyl-CoA is a direct product of the decarboxylation of succinyl-CoA.

TABLE 2

QUANTITIES OF PROPIONHYDROXAMIC ACID FORMED BY A DEPLETED EXTRACT BY THE ACTIVATION OF PROPIONATE AND THE DECARBOXYLATION OF SUCCINATE

INCUBATION PERIOD, MIN.	5 μM PROPIONATE AS SUBSTRATE	50 μM SUCCINATE AS SUBSTRATE	
	μM PROPIONHYDROXAMIC ACID	μM PROPIONHYDROXAMIC ACID	μM CO_2
30	0.03	0.55	0.58
45	0.06	0.77	0.82
60	0.20	0.88	0.93

Reaction mixture contained: 800 μM hydroxylamine, 50 μM phosphate buffer pH 7.3, 10 μM glutathione, 10 μM $MgCl_2$, 100 μM NaF, 10 μM ATP, 36 units CoA; incubated at 30°C. under pure N_2. Values expressed in μM/10 mg. extract protein/hour.

TABLE 3

ACYLATION OF SULFANILAMIDE BY A DEPLETED EXTRACT

μM ATP ADDED	UNITS CoA ADDED	SULFANILAMIDE ACYLATED, μG./10 MG. EXTRACT PROTEIN/HOUR
0	0	0
0	18	2
10	0	2
10	18	10
10	36	12
20	18	22

Reaction mixture contained: 50μM TRIS buffer pH 8.0, 0.2 ml. pigeon liver preparation, 50 μM succinate, 100 μg. sulfanilamide, 10 μM glutathione, 100 μM NaF, 10 μM $MgCl_2$; incubated at 30°C. under pure N_2.

Reversibility of the decarboxylation reaction was tested by incubating an extract with propionate and bicarbonate. The formation of succinate was revealed by the reduction of 2,6-dichlorophenol indophenol under the influence of partially purified succinoxidase. Addition of malonate inhibited dye reduction, as did omission of bicarbonate from the mixture. These observations eliminate oxidation of propionate or other substances present in the extract as the cause of the reduction. On the other hand, addition of ATP and CoA greatly increased the reduction; this was not the case when succinate was used as substrate.

Formation of CoA compounds in extracts: Because the evidence for succinyl-CoA production by phosphotrans-succinylase[1] is indirect, an attempt was made to determine whether acylation of sulfanilamide, considered specific for acyl-CoA derivatives, can occur in extracts provided with ATP, CoA, succinate, sulfanilamide, and "acylating enzyme."[2]

The results of these experiments show unequivocally that sulfanilamide acylation occurs (table 3), though only to a small extent. If succinyl-CoA is the acylating agent, this is readily understandable since this substance is also rapidly decarboxylated; the acylating enzyme must therefore compete with decarboxylase for its substrate. In agreement with this explanation is the fact that dialyzed extracts, with reduced decarboxylase activity, cause a larger sulfanilamide acylation. Moreover, in a reaction mixture incubated at pH 8.0, considerably above the pH optimum for decarboxylation, more sulfanilamide disappears, although this may be partly the result of more favorable conditions for the action of the acylating enzyme.

Both ATP and CoA are essential for sulfanilamide acylation; a small amount of CoA suffices for maximum effect, but a substantial increase in sulfanilamide disappearance is caused by augmenting the ATP supply. Regeneration of CoA as a consequence of the transfer of the acyl moiety of an acyl-CoA compound to sulfanilamide readily accounts for the former; the ATP effect is discussed in more detail below.

Mechanism of succinyl-CoA formation: Small amounts of ATP and CoA in extracts of *M. lactilyticus* permit the decomposition of large quantities of succinate to propionate and carbon dioxide.[1] If this process involved the decarboxylation of succinyl-CoA with the liberation of free CoA, the need for only catalytic amounts of the coenzyme would be understandable. However, the situation with respect to ATP is different; succinyl-CoA formation exclusively by way of succinyl phosphate, or by some other mechanism involving both ATP and CoA, should require a quantity of ATP equivalent to that of succinate decomposed, since it is difficult to conceive of a regeneration of ATP from ADP, or AMP, and inorganic phosphate coupled with a simple decarboxylation.

Because traces of ATP suffice for an extensive succinate decomposition, a mechanism of succinyl-CoA formation independent of ATP appeared probable. Such a mechanism would be provided by a decarboxylation of succinyl-CoA with the liberation of an acyl-CoA compound which could react with free succinate to regenerate succinyl-CoA through the mediation of a CoA transphorase. A reaction of this type has been postulated to account for the activation of propionate[12] and formate[13] by extracts of *Clostridium kluyveri* in the presence of acetyl phosphate and CoA.

Formation of propionyl-CoA by the decarboxylation of succinyl-CoA seemed probable; hence the occurrence of a transphorase reaction between

propionyl-CoA and succinate was investigated. Depleted extracts, able to decarboxylate succinyl-CoA but not free succinate, were incubated with succinate and a small amount of propionyl-CoA. As seen from Table 1, exp. 2, rapid decarboxylation occurred, and the rate of carbon dioxide evolution exceeded that from succinate in extracts supplemented with ATP and CoA.

In view of these results it seemed possible that other CoA compounds, e.g., acetyl-CoA, would likewise serve as CoA donor. Acetyl-CoA was not added as such, but generated in the extract from acetyl phosphate and CoA through the action of phosphotransacetylase whose presence had been established by tests on the arsenolysis of acetyl phosphate and acetylation of sulfanilamide. Table 1, exp. 3, shows that depleted extracts can decarboxylate succinate if supplemented with acetyl phosphate and CoA.

The following experiment provides more direct evidence for succinyl-CoA formation from succinate, acetyl phosphate, and CoA. Depleted extracts, incubated with succinate, acetyl phosphate, and CoA, yield three hydroxamic acids upon the addition of hydroxylamine; these have been identified as acet-, propion-, and succinhydroxamic acids. The latter is formed by the above reaction mixture only if CoA is added, and since in the presence of both acetyl phosphate and CoA, succinate is decarboxylated, the formation of activated propionate could be expected. It should be noted that if hydroxylamine is added at the start of the incubation period, CoA transphorase action cannot be demonstrated and only acethydroxamic acid is found in the above reaction mixture.

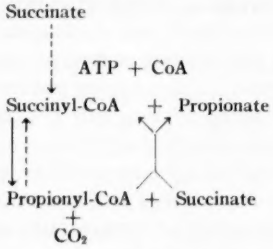
Incubation of depleted extracts with acetyl phosphate, CoA, and propionate, followed by the addition of hydroxylamine, yields propionhydroxamic acid, as well as acethydroxamic acid, showing that CoA transfer can also occur between acetyl-CoA and propionate. Activated formate is produced by substituting formate for propionate in the above reaction mixture. The quantity of succin- and propionhydroxamic acids found in these experiments is far greater than the available amount of CoA, suggesting that phosphate derivatives are formed from the CoA compounds by the action of phosphotrans-succinylase and -propionylase.

The effect of propionyl-CoA and acetyl-CoA on the decarboxylation of succinate, and the evidence for the formation of activated succinate from succinate and acetyl-CoA indicate that generation of succinyl-CoA by CoA transfer can occur. Hence a decarboxylation of succinyl-CoA with the production of an acyl-CoA compound provides a simple mechanism for the continued formation of succinyl-CoA independent of ATP. The experiment summarized in table 2 permits the conclusion that propionyl-CoA is this compound, rather than a one-carbon CoA compound.[14]

In the absence of acyl-CoA compounds, the initial formation of succinyl-CoA must, of course, proceed by other means. That succinate can be

decarboxylated in the presence of CoA and ATP shows that this can be accomplished through a reaction in which ATP takes part, either through the primary formation of succinyl phosphate,[1] or by a different type of reaction involving both ATP and CoA. Such a reaction could result in the concomitant formation of ADP and inorganic phosphate, as described for the activation of succinate by heart preparations,[15] or of AMP and pyrophosphate, as described for the activation of acetate by yeast preparations.[4] Since pyrophosphatase-free extracts of *M. lactilyticus*, incubated with succinate, ATP, CoA, and hydroxylamine[4] showed an increase in inorganic phosphate and gave negative tests for pyrophosphate, it must be concluded that an appreciable quantity of succinyl-CoA is not formed by the latter activation mechanism.

The results of the experiments to date indicate that the decomposition of succinate could be schematically represented by the following diagram:



Theoretically the formation of an infinitely small amount of succinyl-CoA suffices to initiate the decarboxylation. In the presence of hydroxylamine, or of sulfanilamide and an "acylating enzyme," the acyl-CoA compounds of the cycle are partly drained off. Thus the cycle is interrupted, and larger quantities of ATP are required for the primary synthesis of succinyl-CoA.

The diagram indicates that succinate formation by a reversal of the decarboxylation reaction would require carbon dioxide and propionyl-CoA. However, this synthesis of succinate may be considered to be analogous to that of acetoacetate from acetate. It has been established that the latter requires two "active" acetate units, in the form of acetyl-CoA.[16] Hence it appears reasonable to suggest that the synthesis of succinate, too, may be possible only if both propionyl-CoA and an activated one-carbon unit are available. This view receives support from the experiments of Delwiche, *et al.*[14] The effect of ATP and CoA on the synthesis of succinate by extracts of *M. lactilyticus* might be attributed to their involvement in the production of both these "active" building blocks.

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GENERALIZED MATHEMATICAL RELATIONSHIPS FOR POLYPEPTIDE CHAIN HELICES. THE COORDINATES OF THE II *HELIX**

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The formulation of polypeptide chain configurations is concerned principally with intra-chain packing and interactions between atoms in neighboring residue units of the skeletal backbone chain $(—C\cdot CO\cdot NH\cdot—)_n$. The folded or coiled configurations are held together by intra-chain (CO·HN) hydrogen bonds between residues. In helical configurations the residues are structurally equivalent; they all have identical intra-chain environments (exclusive of amino acid residue variations). Such configurations, therefore, effectively provide maximal intra-chain hydrogen bonding.

Detailed studies depend on the use of precise dimensions for the poly-

peptide chain. Pauling and Corey have predicted as the fundamental unit a chain residue with dimensions and configuration derived from detailed studies of crystal structure analyses of amides, amino acids, and peptides.[1] On the same basis they have also defined appropriate limits for the hydrogen-bonded oxygen to nitrogen distance and for the angle between the NH and NO vectors.

Only two helical[2, 3] configurations, the α and γ helices, fulfill completely all of the requirements specified by Pauling and Corey. A third helix,

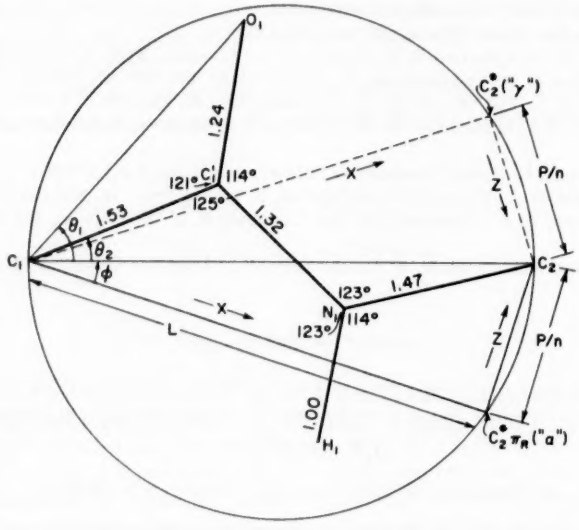

FIGURE 1

Orientation of the chain residues in the "α" and "γ" series. Derivation of the x and z coordinates for the atoms of the first residue. The x and z axial directions in each series are perpendicular and parallel, respectively, to the helical axis.

the π helix,[4] involves so small a deformation (4°) in the intra-chain α carbon bond angle that it also should probably be considered to fulfill their requirements. Deformations of this order have been observed in amino acid structures.[5]

The π helix was discovered while working with three-dimensional scale models which had dimensions slightly different from the Corey-Donohue[1] dimensions first used by Pauling and Corey. In an attempt to determine the best set of atomic coordinates for this structure, an analytical method was developed. The procedure establishes certain relationships between

different polypeptide chain helices which appear to have been confused in the past. The method is rapid and quite general; it may also be useful in the study of non-peptide helical structures where there exists a choice among different values for the independent variables.

In the three helices, α, π, and γ, which satisfy the Pauling-Corey requirements, each residue (—C·CO·NH·C—) is coplanar and lies in one of a zone of planes parallel to and at a common distance from the helical axis. The helices may be described by specifying the carbonyl oxygen to which each NH group is hydrogen bonded. Thus, in the π helix there is a hydrogen bond between one amide group and the fourth amide group beyond it along the chain. This description, however, is inadequate since it overlooks the two fundamentally different ways of coiling a helical structure.

In figure 1 the fundamental dimensions for a single residue —·C_1·$C_1'O_1$·N_1H_1·C_2·— have been drawn to scale for the most recently revised set of values[6] and inscribed in a circle of diameter C_1—C_2. The residue lies in a vertical plane and if one end (C_1) is kept fixed, the residue may be tilted either up or down. If P is the pitch of the final coil, the vertical translation per complete turn, and n is the number of residues per complete turn, then in a given helix this vertical translation per residue is P/n. If the residue is tilted upward so that C_1—C_2^* (solid line) is its horizontal projection (normal to the helical axis), then the CO and NH groups are almost vertical and parallel to the helical axis. A P/n value of 1.14 A was used to construct this figure and over the range P/n = 0.9 → 1.5 A deviations from the vertical are not very large. Tan ϕ = P/nL where L = C_1—C_2^*. The α and π helices are both constructed in this way and we have considered all such helices as belonging to the "α" series.

When the residue is tilted downward so that C_1—C_2^* (broken line) is horizontal then the CO and NH groups are both sharply inclined to the helical axis. The C_1—C_1' and N_1—C_2 bonds are practically horizontal and parallel to C_1—C_2^* over a wide range. The γ helix is constructed in this way and we have considered all such helices as belonging to the "γ" series.

The residue-unit configurations differ markedly between the two series. Direct transformations between members of different series is impossible. Thus, the early suggestion[7] that the γ-helix might represent supercontracted keratin and supercontraction corresponds to a direct transformation $\alpha \rightarrow \gamma$ is theoretically invalid without reference to the comparative instability of either configuration.

Figure 2 (*a*) shows a perspective drawing of the π helix with 4.4 turns and a pitch P = 5.02 A and hence a unit translation per residue P/n = 1.14 A. Each succeeding residue, $C_1(CO·NH)C_2$, $C_2(CO·NH)C_3$, etc.,

lies in a vertical plane parallel to the helical axis and as the plan view, figure 2 (*b*), shows the angle between the two planes is the helical angle $\psi = 360/n$. The angle $180 - \psi$ is the horizontal component of the tetrahedral angle $N_1C_2C_2'$ and so the tetrahedral angle depends on both the number of turns (n) in the configuration and the vertical unit residue translation. Both the plan view (in which the nitrogen positions have been omitted at the cross-over points) and the drawing, illustrate that the CO and NH groups are almost vertical and the angle between the NH and NO vectors is small.

The π helix shown has a right-handed screw axis. The mirror image left-handed helix corresponds to the mirror image of figure 1 with C_1—C_2 plotted from right to left. Looking at the helix upside down corresponds to a horizontal projection C_1^*—C_2 (not drawn) parallel to C_1—C_2^*.

As a helix is wound up, a residue may theoretically form hydrogen bonds with the nth amide group beyond it. Figure 2 shows that for the π helix this corresponds to the chemical sequence,

O.....H
‖ |
C—(NH·C(HR)·CO)$_4$ N

read in either direction. For the "α" series the sequence is therefore

O.....H
‖ |
C—(NH·C(HR)·CO)$_n$ N

with $3n + 4$ atoms in the hydrogen-bonded loop so formed. Bragg, Kendrew and Perutz[8] have identified helices by symbols which indicate the number of turns in a unit repeat (screw symmetry) with subscript giving the number of atoms in the hydrogen-bonded loop. The π helix[4] would thus be written 4.4_{16}. We have avoided this identification where possible, for it leads to ambiguities; the helices 3.6_{13} and 3.7_{13} are both the α helix,[9] whereas 3.6_{14} is a helix of the "γ" series.

Variations in the number of turns per unit repeat are correlated with changes in the "tetrahedral" angle and hydrogen bond length and angle. Minor revisions are therefore likely to be made from time to time. The right-handed helix π_R in the drawing is shown with two of the β carbons of the amino acid residues drawn in (βC_2, βC_4) at the correct position for naturally occurring L amino acids.[10]

The "γ" series residue unit in figure 1 would lead to left-handed helices. In winding up these units so that a residue may form hydrogen bonds with the nth amide group beyond it, the chemical sequence

Fig. 2 (*a*)—Perspective drawing of the right-handed π_R helix. The link-end carbon atoms are numbered consecutively. The β carbon and hydrogen atom positions for L-amino acids are shown attached to C_2 (α) and C_4 (α). Fig. 2 (*b*)—Plan view of π_R helix. The hydrogen positions are shown with broken lines. Nitrogen positions are omitted at the two cross-over points.

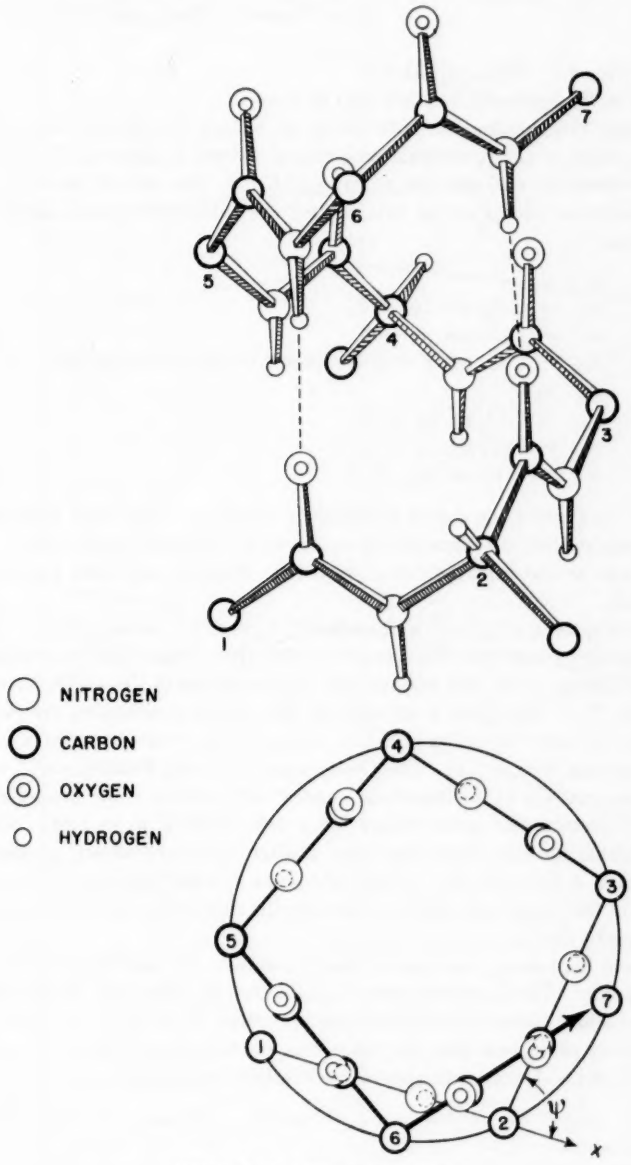

FIGURE 2 (*a*), ABOVE; 2(*b*), BELOW

H.....O is obtained. There are $3(n-1)+5$
| ||
N (C(HR).CO.NH)$_{n-1}$C(HR) C

atoms in the hydrogen-bonded loop so formed.

Atomic coordinates for both series of helices are derived as follows. If the origin of the rectangular coordinate system is taken at C_1 with the x axis along C_1—C_2^* and the z axis parallel to the axis of the helix, the coordinates of atoms in the first residue are calculated from equations of the kind:

$$\begin{aligned} x_{C_1} &= 0 \\ x_{O_1} &= C_1O_1 \cos(\phi + \theta_1) \\ x_{C_1'} &= C_1C_1' \cos(\phi + \theta_2) \qquad (1) \\ x_{C_2} &= C_1C_2 \cos\phi = L \text{ (projected length of the residue)} \\ z_{C_1} &= 0 \\ z_{O_1} &= C_1O_1 \sin(\phi + \theta_1) \\ z_{C_1'} &= C_1C_1' \sin(\phi + \theta_2) \\ z_{C_2} &= C_1C_2 \sin\phi = P/n \qquad (2) \end{aligned}$$

In the "γ" series the z axis direction is changed. The same expressions hold except that the signs of the angles θ_1, θ_2, θ_3 and θ_4 are reversed. For all atoms in the first residue $y = 0$ (the residues are both planar and vertical).

The lengths C_1O_1, C_1C_1', and angles θ_1, θ_2, etc., are constants for a given residue model and may be calculated directly. The x and z coordinates for the atoms of the first residue unit are functions of the vertical rise per residue, P/n. In figure 3 (*a*) and (*b*) the atomic coordinates are plotted for the "α" and "γ" series of helices, respectively, over the range of values of P/n from 0.95 to 2.0. They were prepared using Pauling and Corey's most recent set of fundamental residue dimensions. For example, the broken vertical line gives values for α helix with $P/n = 1.48$. Similar expressions for the Corey-Donohue configuration are shown graphically in figures 4 (*a*) and (*b*). There are some marked differences near the limits of the range explored even though the dimensions of the two models are closely similar.

Planes containing successive linked residues are equidistant from the helical axis. The α carbon atoms $C_1C_2C_3$, etc., lie, therefore, on the surface of a cylinder about the helical axis of radius $R = L/(2 \sin \psi/2)$. In horizontal projection they lie, therefore, on the circumference of a circle (Fig. 2 (*b*)). The coordinates of the rth link-end position are:

$$x_{C_r} = L(0 + 1 + \cos\psi + \cos 2\psi \ldots + \cos(r-2)\psi) \qquad (3a)$$

Fig. 3 (*a*)—Atomic coordinates for the "α" series atom positions plotted as a function of P/n. (Pauling-Corey dimensions.[6])

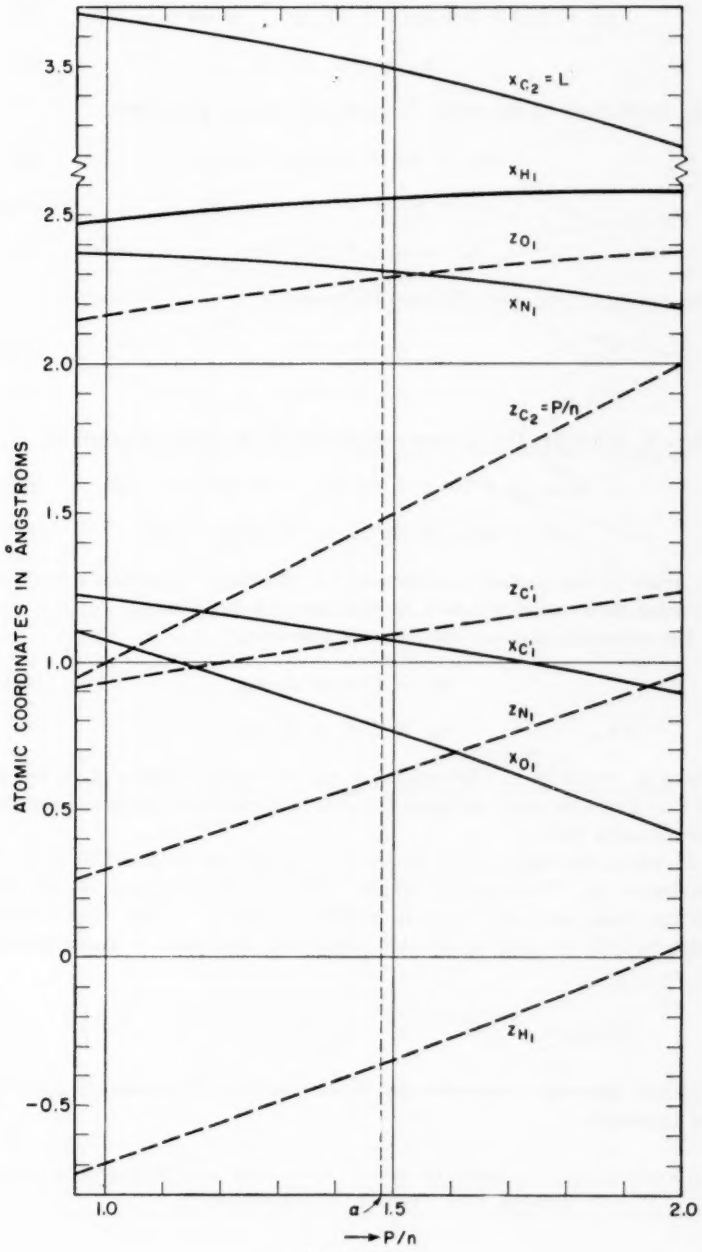
3.5
2.5
2.0
1.5
1.0
0.5
0
-0.5
ATOMIC COORDINATES IN ÅNGSTROMS
$x_{C_2} = L$
x_{H_1}
z_{O_1}
x_{N_1}
$z_{C_2} = P/n$
$z_{C'_1}$
$x_{C'_1}$
z_{N_1}
x_{O_1}
z_{H_1}
1.0
a
1.5
2.0
P/n

FIGURE 3 (*a*)

$$y_{C_r} = L(0 + 0 + \sin\psi + \sin 2\psi \ldots + \sin(r-2)\psi) \quad (3b)$$

$$z_{C_r} = (r-1)P/n \quad (3c)$$

The coordinates of the point P_r in the rth link is given by:

$$x_{P_r} = x_{C_r} + x_{P_1}\cdot\cos(r-1)\psi \quad (4a)$$

$$y_{P_r} = y_{C_r} + x_{P_1}\cdot\sin(r-1)\psi \quad (4b)$$

$$z_{P_r} = (r-1)P/n + z_{P_1} \quad (4c)$$

The equations (3a) and (3b) may be rewritten:

$$x_{C_r} = L\cdot u_r \quad (5a)$$

$$y_{C_r} = L\cdot v_r \quad (5b)$$

where u_r and v_r are the unitary coordinates of the link-end positions.

$$u_r = (1 + \cos\psi + \cos 2\psi \ldots + \cos(r-2)\psi) \quad (6a)$$

$$v_r = (\sin\psi + \sin 2\psi \ldots + \sin(r-2)\psi) \quad (6b)$$

In figure 5 the unitary coordinates for successive α carbon atoms are recorded for r values of 2 to 6 over the range $n = 3.0$ to 5.5.

The equations (4a) and (4b) may be rewritten:

$$x_{P_r} = L.u_r + A_r\cdot x_{P_1} \quad (7a)$$

$$y_{P_r} = L\cdot v_r + B_r\cdot x_{P_1} \quad (7b)$$

where $A_r = \cos(r-1)\psi$ and $B_r = \sin(r-1)\psi$. Values of A_r and B_r for $r = 2$ to 6 are given in figure 6 plotted against n the number of residues per complete turn.

Atomic coordinates of the atoms N_1, C_2, C_2' are required in order to determine the "tetrahedral" angle. The direction cosines of the two vectors N_1C_2 and C_2C_2' may be readily evaluated. The "tetrahedral" angle $N_1C_2C_2' = \chi$ is given by the usual cosine expression. This simplifies to

$$\cos\chi = -\left\{\frac{(A_2\cdot x'_{C_1})\cdot(L - x_{N_1}) + z'_{C_1}\cdot(P/n - z_{N_1})}{N_1C_2\cdot C_2C_2'}\right\} \quad (8)$$

Similarly the angle τ between the N_r—O_1 and N_r—H_r vectors is given by the equation:

Fig. 3 (b)—Atomic coordinates for the "γ" series atom positions plotted as a function of P/n. (Pauling-Corey dimensions.[6])

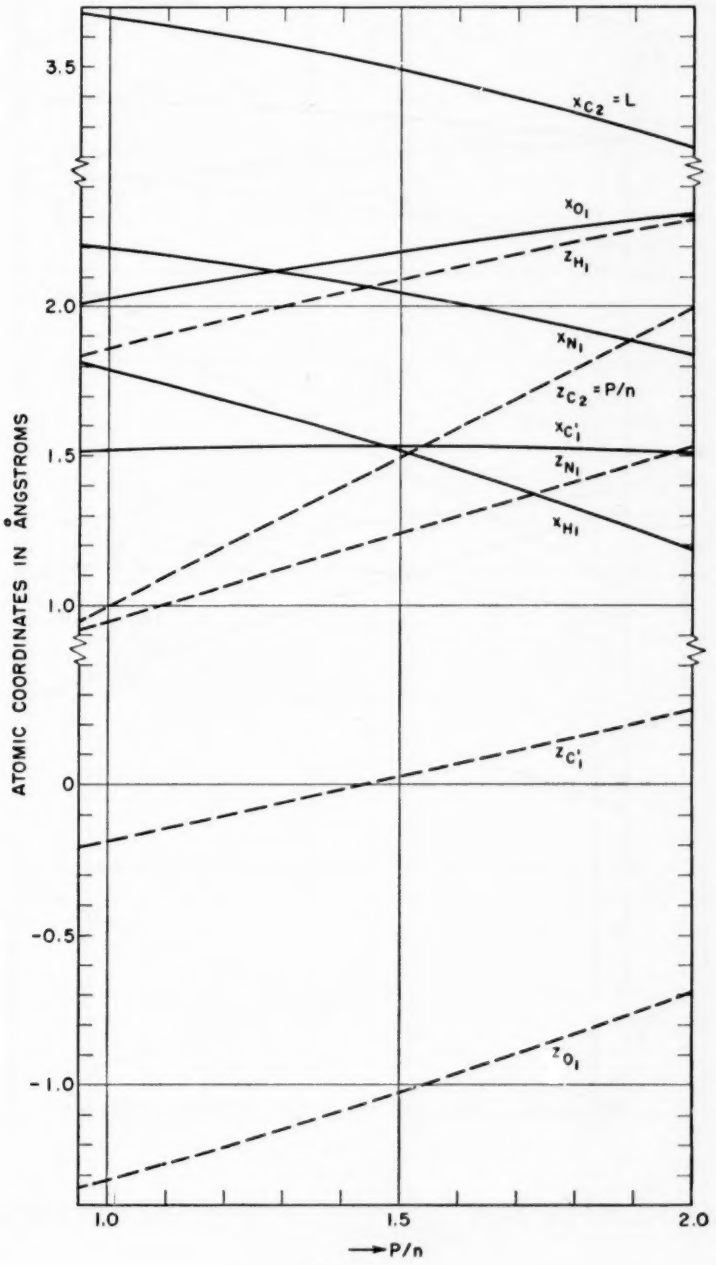
3.5
2.0
1.5
1.0
0
-0.5
-1.0
ATOMIC COORDINATES IN ÅNGSTROMS
$x_{C_2} = L$
x_{O_1}
z_{H_1}
x_{N_1}
$z_{C_2} = P/n$
$x_{C_1'}$
z_{N_1}
x_{H_1}
$z_{C_1'}$
z_{O_1}
1.0
1.5
2.0
P/n

FIGURE 3 (*b*)

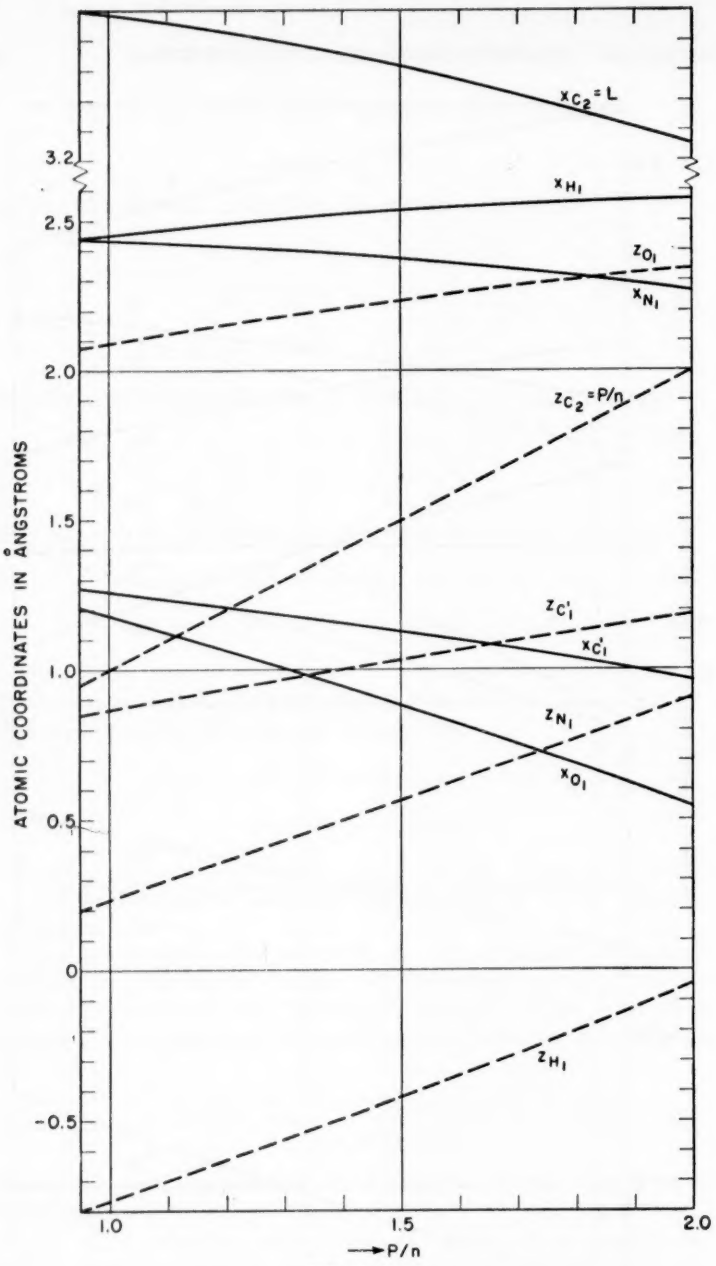

FIGURE 4 (*a*)

Atomic coordinates for the "α" series atom positions plotted as a function of P/n. (Corey-Donohue dimensions.[1])

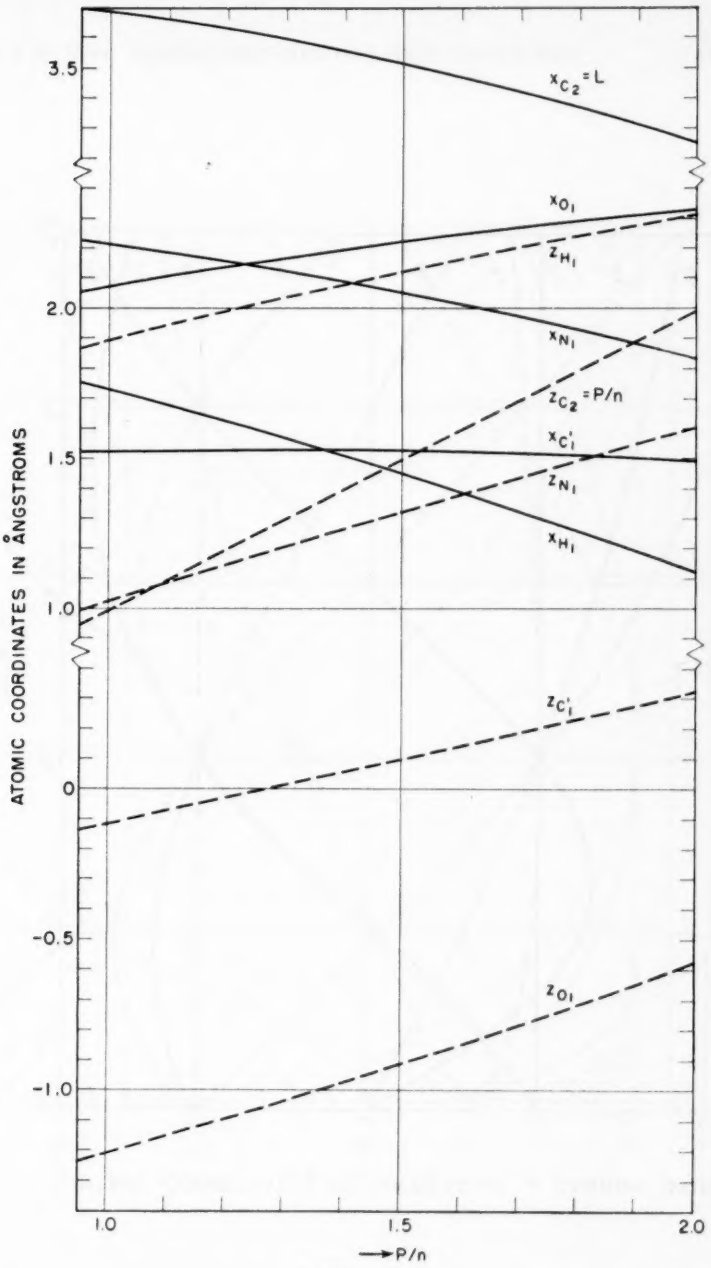

FIGURE 4 (*b*)

Atomic coordinates for the "γ" series atom positions plotted as a function of P/n. (Corey-Donohue dimensions.[1])

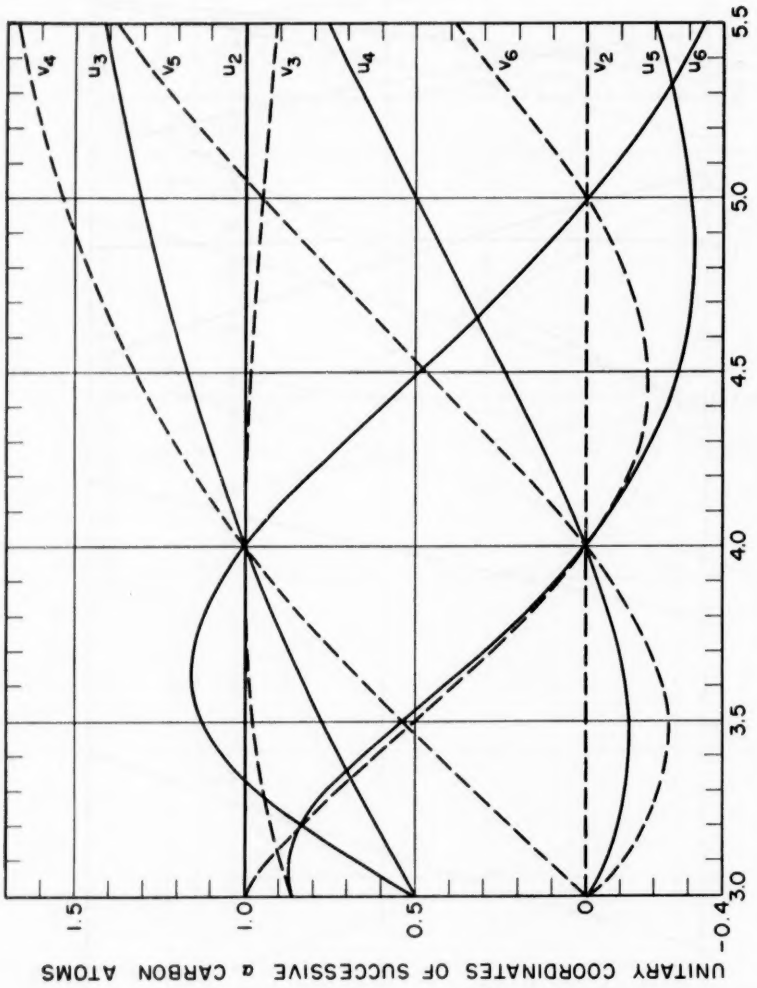

FIGURE 5

Unitary coordinates for successive α carbon atoms.

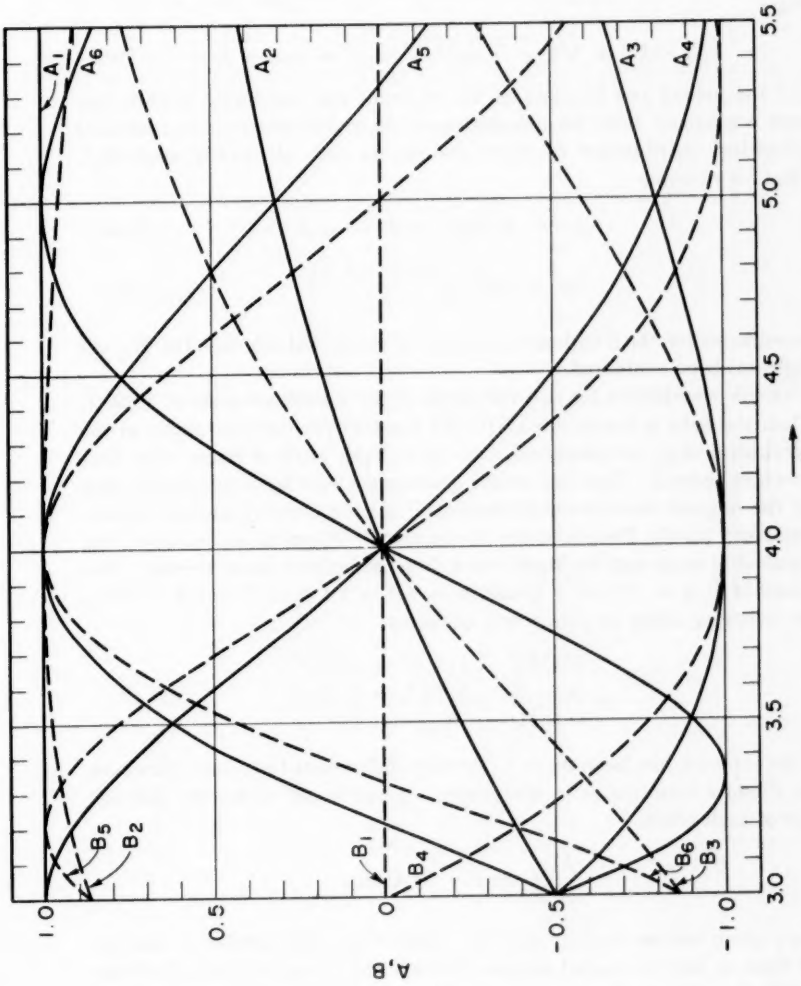

FIGURE 6

Values of $A_r = \cos(r-1)\psi$ and $B_r = \sin(r-1)\psi$.

$$\cos\tau = \frac{(x_{N_r} - x_{O_1})(x_{N_r} - x_{H_r}) + y_{N_r}(y_{N_r} - y_{H_r}) + (z_{N_r} - z_{O_1})(z_{N_r} - z_{H_r})}{N_rH_r \cdot N_rO_1} \quad (9)$$

where

$$N_r - O_1 = \sqrt{(x_{N_r} - x_{O_1})^2 + (y_{N_r})^2 + (z_{N_r} - z_{O_1})^2} \quad (10)$$

If the helical axis is taken as the origin of the coordinate system (the most convenient form for calculation of the radial distribution function) cylindrical coordinates[3] (ρ, δ) of the atoms may be readily evaluated. Thus for example

$$\rho_{N_1} = \sqrt{(x_{N_1})^2 + R^2 - x_{N_1}L} \quad (11a)$$

$$\delta_{N_1} = \sin^{-1}\left(\frac{x_{N_1} \cdot \cos\psi/2}{\rho_{N_1}}\right) \quad (11b)$$

Coordinates for the β carbon atom may be easily derived once the $N_1C_2C_2'$ angle has been evaluated.

Atomic coordinates for two variations of the π helix are given in table 1. When the helix is constructed with 4.3 residues per turn the strain in the tetrahedral angle is minimized, but the $O_1N_5H_5$ angle is larger than that shown in figure 2. Series of atomic coordinates have been calculated using (*a*) the original scale model dimensions,[11] (*b*) the Corey-Donohue[1] dimensions, and (*c*) the Pauling-Corey dimensions.[6] From these, values of the tetrahedral angle and hydrogen bond dimensions have been derived. For values of $P/n = 1.14$ to 1.15 and $n = 4.3$ to 4.4 (i.e., $P = 4.9$ to 5.06), the following range of values was obtained.

$\angle N_1C_1C_2'$	113.5° → 115.5°
$O_1 - N_5$	2.75 A → 2.83 A
$\angle O_1N_5H_5$	3.5° → 18°

No attempt has been made to develop differential functions; however, the x and z coordinates in the graphs, figures 3 and 4, are the differentials of each other.

$$\frac{\delta}{\delta x} A\cos x = -A\sin x$$

For a given residue configuration the pitch P and the number of residues per turn, n, may be varied independently. Once a set of values has been chosen it is obvious what type of adjustment is needed to improve the solution. A graphical method[12] has also been devised which permits extremely rapid trial and error determinations of the three critical variables.

Four helices of the "α" class have been described. "α"$_{n=1}$ corresponds

to the 2_7 or α_{11} configuration proposed by Huggins,[13] Zahn,[14] and Mizushima.[15] Examination of figure 1 shows that in this helix the $O_1N_2H_2$ angle will be large for the values of P/n required. "α"$_{n=2}$ corresponds to the 3.0_{10} helix suggested by Taylor[16] and Huggins. "α"$_{n=3}$ is the α

TABLE 1

ATOMIC COORDINATES, AND VALUES FOR $N_1C_2C_2'$, N_5—O_1, AND $O_1N_5H_5$; x, y and z in A

	x	y	z
	$n = 4.3$, $P = 4.90$, $P/n = 1.14$		
C_1	0	0	0
C_1'	1.18	0	0.98
O_1	0.99	0	2.20
N_1	2.36	0	0.39
H_1	2.51	0	−0.60
C_2	3.62	0	1.14
Helical axis	1.81	2.02	..
	$\angle N_1C_2C_2'$	113.5°	
	N_5—O_1	2.79 A	
	$\angle O_1N_5H_5$	17.5°	
	$n = 4.4$, $P = 5.06$, $P/n = 1.15$		
C_1	0	0	0
C_1'	1.18	0	0.98
O_1	0.98	0	2.21
N_1	2.36	0	0.40
H_1	2.51	0	−0.59
C_2	3.62	0	1.15
Helical axis	1.81	2.09	..
	$\angle N_1C_2C_2'$	115°	
	N_5—O_1	2.80 A	
	$\angle O_1N_5H_5$	10.5°	
	"α"$_{n=5}$, $n = 5.2$, $P = 4.95$, $P/n = 0.95$		
C_1	0	0	0
C_1'	1.23	0	0.91
O_1	1.10	0	2.15
N_1	2.38	0	0.27
H_1	2.48	0	−0.73
C_2	3.68	0	0.95
Helical axis	1.84	2.66	..
	$\angle N_1C_2C_2'$	122°	
	N_5—O_1	2.93 A	
	$\angle O_1N_5H_5$	17°	

helix and "α"$_{n=4}$ is the π helix. "α"$_{n=5}$ has been studied by us. The horizontal component of the $N_1C_2C_2'$ angle is $180–360/(5 + \delta)$ where δ is fractional, and is, therefore, itself of the order of 110°. In the "α" series the vertical component of this angle is considerable. For $n = 5.2$, $P =$

4.95, and the Pauling-Corey model, figure 1, the $N_1C_2C_2'$ angle is 122° (table 1). This deformation is large. However, as the range of values for the helix shows, slight changes in the model and in the values for P and n, may lead to considerable differences in the tetrahedral angle derived. Hydrogen bond length and angle are reasonable for the structure. All the members of the group $"\alpha"_{n=1} \rightarrow "\alpha"_{n=5}$ are acceptable if the restrictive criteria are somewhat relaxed. Transformation between different configurations may be accomplished by simultaneously decreasing or increasing the wind up angle, and either reducing or increasing the vertical tilt per unit residue.

Four helices of the "γ" series have been described. In this series the angle $N_1C_2C_2'$ is almost equal to its horizontal component $180 - \psi$. $"\gamma"_{n=2}$ (2_8)[13] and $"\gamma"_{n=3}$ (the 11 atom ring)[17] are therefore completely excluded for the coplanar configuration. In $"\gamma"_{n=4}$ this angle is still rather small. For the variant with 4.3[18] residues per turn the horizontal component is 96.3°. $"\gamma"_{n=5}$ is the γ helix. $"\gamma"_{n=6}$ and higher members of this series give "tetrahedral" angles which are much too large.

The relative stabilities of polypeptide chain helices have been evaluated in terms of structural features which are not considered here. Pauling and Corey[19] have estimated, for the α and γ helices, the strain involved in possible "unfavorable orientation" about the N_1C_2 and C_2C_2' bonds, and incomplete van der Waals' stabilization.[9] Robinson and Ambrose[20] and more recently Donohue[18] have both made further estimates of the qualitative or semiquantitative agreement between certain helices and a wide range of restrictive criteria. The fundamental residue dimensions used are not specified in detail for either study. Both groups of investigators have used models different from the Pauling-Corey residue unit, and considerable latitude, including the introduction of slight distortions from the planar amide configuration, has been permitted in adjusting the residue dimensions to improve the agreement with their theoretical requirements.

The graphs reproduced in this paper permit the rapid variation of helical configurations based on a particular model, but must be redrawn for each new residue model. The amount of variation to be expected is illustrated by comparison of Figs. 3 and 4. These graphs are for a residue lying wholly in one vertical plane parallel to the helical axis. While this restriction can be easily relaxed in any particular case, once values of P/n and n have been chosen, systematic allowance for lack of coplanarity would involve the construction of families of graphs. This elaboration did not appear justified at this time.

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CONDUCTANCE AND VISCOSITY OF CONCENTRATED AQUEOUS SALT SOLUTIONS AT 50.5°

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Our knowledge of the electrical conductance and the viscosity of concentrated salt solutions is very limited. Campbell and Kartzmark[1] have measured the conductance and the viscosity of solutions of silver nitrate and ammonium nitrate in water at 95° up to saturation. Seward[2] has measured the conductance and viscosity of solutions of tetrabutylammonium picrate in butanol at 93° over the complete range from pure solvent to pure fused salt; Strong[3] has measured the conductance of some quaternary ammonium salts in benzene at 25° up to fairly high concentration but viscosity data are for the most part lacking. Moessen and Kraus[4] have measured the conductance of solutions of tetrabutylammonium bromide and trimethylammonium chloride in bromine at 25° to fairly high concentrations but there are no viscosity data for these solutions. The same is true of potassium iodide in iodine at 140°C. which was measured by Lewis and Wheeler.[5]

Cesium formate has been reported to be soluble to the extent of one mole of salt to one-half mole of water at 50°.[6] However, this datum is in error; the limit of solubility is one mole of salt to approximately two moles of water. However, potassium formate is soluble to the extent of approximately one mole of salt to 1.1 mole of water at 50°. The conductance and viscosity of solutions of these two salts have been measured at 50.5° to near saturation. Solutions of the potassium salt were measured to lower concentration in order to approximate the value of Λ_0.

In the preparation of cesium formate, cesium chloride was converted to cesium nitrate by repeated treatment with nitric acid. After repeated recrystallizations, the nitrate was converted to carbonate by treatment with oxalic acid in the presence of a small amount of water and subsequent calcination. The carbonate was converted to formate by neutralization with formic acid and evaporating to dryness. The salt was recrystallized by dissolving it in 96% alcohol and adding ether to precipitate the desired proportion of salt. The final product was dried in vacuo at 150° for 4–5 hours, m.p. 264°C. (corr.). The nitrate, when tested spectroscopically, showed no traces of metallic elements other than cesium.

Potassium formate was prepared by neutralizing potassium carbonate with formic acid and evaporating to dryness. The salt was recrystallized from 96% alcohol on addition of ether. The product was dried in vacuo at 150° for 8 to 10 hours. This material was found to contain 1.5% water.

The water could be completely removed by regrinding the salt and heating in vacuo at 150° for 10 hours. When necessary, operations were carried out in a dry-box.

The solutions were made up in the cells in which their conductance was measured. Three cells of different constants were employed. Two of these were of the Erlenmeyer type with the electrode chamber attached to the outside of the flask. These cells were similar to those described by Daggett, Bair, and Kraus,[7] although much smaller. The third cell consisted of two cylindrical tubes which were joined at the bottom by a 55-mm. length of smaller tubing of approximately 5 mm. diameter. The cylindrical cells were closed by ground glass caps which carried tubes at the ends of which were sealed platinum electrodes that normally projected into the solution. The cell constant of this cell was 38.430 and was independent of the precise setting of the caps or the volume of solution in the cell. This cell was provided with stopcocks so that the solution could be forced from one arm to the other by air pressure for the purpose of mixing. The other two cells had constants of 0.3081 and 10.669.

Cell No. 1 was of approximately 300 cc. capacity and was used for measuring the most dilute solutions. A known weight of salt was introduced and weighed quantities of water were added successively. When the cell was full, the greater portion of the solution was withdrawn into a weight pipette and a new series of additions of water was begun. Cell No. 2 had a capacity of approximately 800 cc. and was used for intermediate concentrations. Measurements could be made with a minimum volume of 50 cc. of solution. The desired concentration range could be covered by merely adding known weights of water to a known weight of salt. Cell No. 3 had a capacity of approximately 100 cc. and measurements could be made with a very small volume of solution. The desired concentration range could be covered by merely adding successive weights of water.

To determine the densities, known solutions were made up by adding known weights of water to known weights of salt in a large weighing bottle. Samples of these solutions were transferred to a pycnometer which had been calibrated at 50.5°. Since the density of the solutions varies slightly from linearity, data are given for densities at a series of concentrations.

The density of dry cesium formate was determined by weighing the dehydrated salt in a pycnometer under hexane. From the weight of hexane and its density and the weight of salt, the density of the salt is readily computed. Assuming the density of hexane to be 0.6550, the density of the salt was found to be 2.99. The density of potassium formate was taken to be 1.91.[8]

Viscosities were measured with a modified Ostwald viscometer. The viscometer was provided with a ground glass cap and solutions were made up in the viscometer. The instrument was calibrated with water and the

time of efflux was determined as a function of volume of water. The viscometer with its contents was weighed on a sensitive balance. Concentration could be changed by addition of water and further dilution could be made by withdrawing a known weight of solution and then making further addition of water.

Densities of solutions in water at 50.5° are given for cesium formate and potassium formate in tables 1A and 1B, respectively. Molal concentrations appear in column 1, molar concentrations in column 2, and densities in column 3.

TABLE 1

DENSITY OF FORMATE SOLUTIONS AT 50.5°C.

(A) CESIUM FORMATE		(B) POTASSIUM FORMATE	
MOLAR CONCENTRATION, C	DENSITY	MOLAR CONCENTRATION, C	DENSITY
0.00	0.9878	0.00	0.9878
1.193	1.142	3.967	1.160
2.414	1.303	5.643	1.232
4.892	1.613	9.452	1.392
9.068	2.147		

TABLE 2

CONDUCTANCE OF FORMATE SOLUTIONS AT 50.5°C.

(A) CESIUM FORMATE		(B) POTASSIUM FORMATE	
MOLAR CONC.	EQUIVALENT COND.	MOLAR CONC.	EQUIVALENT COND.
10.13	24.80	6.503	63.03
10.06	24.84	3.492	96.17
9.034	33.18	10.45	30.82
6.913	54.33	8.602	44.24
4.629	81.78	15.52	9.780
0.8104	138.1	13.66	14.65
0.5430	145.8	0.5999	142.2
0.3055	153.5	0.2722	154.2
3.231	98.84	0.1543	161.6
2.437	109.5	2.404	110.4
1.785	118.9	1.325	126.4
1.400	125.6	0.07471	168.7
		0.01973	176.6
		0.01038	179.8

Conductance data for the cesium and potassium salts are presented in tables 2A and 2B, respectively. Molar concentrations are given in column 1 and equivalent conductances in column 2.

Viscosities for solutions of cesium formate and potassium formate are given in tables 3A and 3B, respectively. Molar concentrations are given in column 1, relative viscosities in column 2, and absolute viscosities in centipoises in column 3.

In tables 4A and 4B are presented values of the conductance-viscosity product for the cesium and potassium salts, respectively, at rounded molar concentrations. In column 2 are given values of the ratios of moles of salt per mole of water. Values of equivalent conductance and of viscosity

TABLE 3

VISCOSITY OF FORMATE SOLUTIONS AT 50.5°C.

(A) CESIUM FORMATE		(B) POTASSIUM FORMATE	
MOLAR CONC.	$\eta \times 10^2$ POISES	MOLAR CONC.	$\eta \times 10^2$ POISES
9.605	2.612	13.95	5.173
8.203	1.809	8.224	1.463
6.834	1.338	6.090	1.028
3.989	0.8500	4.199	0.8277
2.808	0.7313	3.125	0.7296
5.819	1.117	15.64	8.223
1.784	0.6533	12.26	3.403
0.7496	0.5847	10.38	2.216
		1.516	0.6245

TABLE 4

CONDUCTANCE-VISCOSITY PRODUCT OF FORMATES AT 50.5°C.

MOLAR CONC.	MOLES H_2O PER MOLE SALT	CONDUCTANCE	$\eta \times 10^2$ POISES	$\Lambda\eta$-PRODUCT
		(A) Cesium Formate		
0.00	...	192.9	0.5449	1.051
0.25	...	157.2	0.560	0.879
1.00	0.01893	134.0	0.610	0.817
2.00	0.04080	115.5	0.670	0.774
4.00	0.09141	88.8	0.840	0.746
7.00	0.1981	52.8	1.37	0.723
9.00	0.3018	32.7	2.22	0.726
10.00	0.3704	25.0	2.95	0.738
		(B) Potassium Formate		
0.00	...	187.9	0.5449	1.024
0.50	...	145.4	0.565	0.822
1.00	0.01889	132.8	0.593	0.788
3.00	0.06223	102.3	0.725	0.742
5.00	0.1150	78.8	0.905	0.713
7.00	0.1802	58.3	1.165	0.683
9.00	0.2634	41.0	1.680	0.689
13.00	0.5176	16.8	4.09	0.687
14.00	0.6146	13.6	5.19	0.706
15.50	...	9.9	7.89	0.781

appear in columns 3 and 4, respectively, and values of the conductance-viscosity product in column 5.

In figure 1 are shown plots of the equivalent conductance of cesium and potassium formates as a function of the square root of molar concentration. At low concentrations (not shown on figure), the conductance of the ce-

sium salt parallels and lies several Λ-units above that of the potassium salt. This is due to the fact that the conductance of the cesium ion is several Λ-units higher than that of the potassium ion. According to Noyes and Falk,[9] the conductance of the cesium ion at 18°C. is 3.5 Λ-units greater than that of the potassium ion. These early measurements are not of high pre-

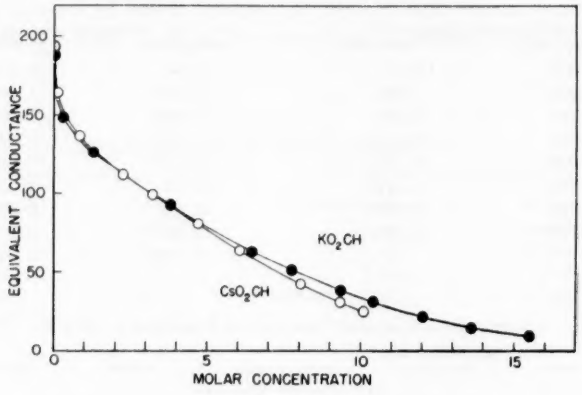

FIGURE 1

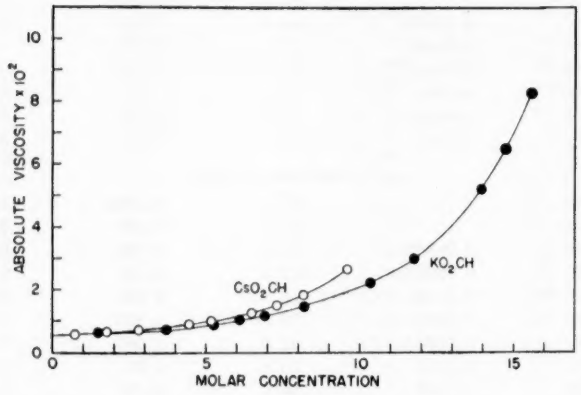

FIGURE 2

cision and the precise difference in the conductance of the cesium and potassium ions at 50.5° remains uncertain. However, it is safe to assume that the conductance of the cesium ion is the greater. We have extrapolated the conductance of potassium formate to zero concentration, using the $\Lambda - \sqrt{C}$ plot. We obtained the value 187.9 at 50.5°.

Using the data of Harned and Owen,[10] for the conductance of the formate ion at 25° and those of Auerbach and Zeglin[11] for the conductance of sodium formate at 18°, we obtain a value of 76 for the conductance of the formate ion on extrapolating linearly to 50.5°. From the equations of Harned and Owen we obtain a value of 112.1 for the conductance of the potassium ion at 50.5°. Thus, we have the value of 188.1 for the limiting con-

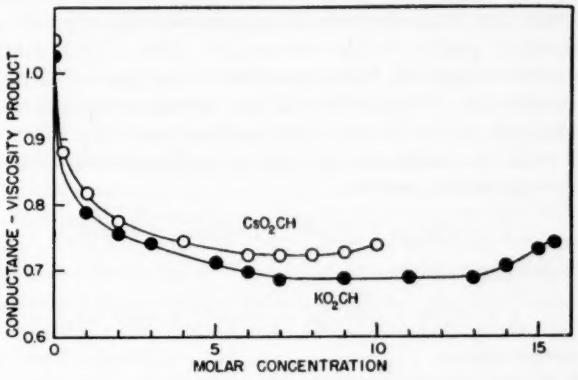

FIGURE 3

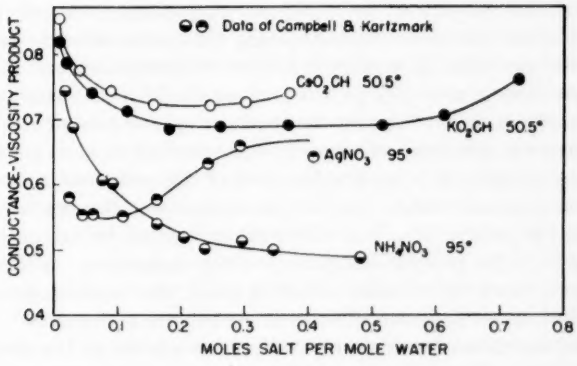

FIGURE 4

ductance of potassium formate at 50.5° in good agreement with our extrapolated value.

At higher concentrations, the conductance curves of the two salts intersect at a concentration of approximately 2.5 molar. At still higher concentrations the curves diverge increasingly.

At the highest concentrations measured, the conductance of cesium and

potassium formates are 24.80 and 9.38, respectively. At a concentration of 10 molar, the conductances are 25.0 and 33.7, respectively.

While the conductance of electrolytes at low concentrations is governed by the interaction between their ions, in concentrated solutions conductance is mainly dependent on the viscosity of their solutions. In figure 2 are shown values of the absolute viscosity of solutions of the two formates as a function of molar concentration. As may be seen from the figure, viscosity increases markedly with concentration in the case of both salts, being somewhat greater for the cesium salt. For a 10-M solution, the viscosity of the cesium salt is 5.4 times that of the pure solvent. For the potassium salt it is 3.9 times that of the solvent at 10 molar and 14.8 times that of the solvent at 15.5 molar concentration. Up to a concentration of $C = 12$, the measured viscosities in centipoises may be reproduced reasonably well by the equation:

$$\eta = 0.545 + 0.0755C - 0.0135C^2 + 0.003C^3$$

for cesium formate, and by

$$\eta = 0.545 + 0.0473C - 0.004C^2 + 0.00107C^3$$

for potassium formate.

To a first order of approximation, the conductance of a completely dissociated electrolyte at different concentrations is proportional to the reciprocal of the viscosity of the solutions. Accordingly, we may compensate for the viscosity effect by multiplying the conductance by the viscosity. If the electrolyte is completely dissociated into its ions and the nature of the ions undergoes no change, the product should remain constant.

In figure 3 are shown values of the conductance-viscosity product for the two formates as a function of concentration to near saturation. The product for the cesium salt is greater than that of the potassium salt over the entire concentration range. At low concentrations, the product for the cesium salt is greater than that of the potassium salt by an amount that corresponds to the greater conductance of the cesium ion. At lower concentrations, when the viscosity effect is small, the conductance falls off sharply with increasing concentration in accordance with theory. As concentration increases, the two curves diverge as a result of the greater viscosity of the solutions of the cesium salt. At a concentration of 5 molar, the curves flatten out over a considerable concentration range. The product for cesium formate appears constant between 6 and 9 molar; for potassium formate it is constant between 7 and 13 molar. Over these ranges of concentration the number of moles of water per mole of salt decreases from 6 to 3 for the cesium salt and from 5 to 2 for the potassium salt.

It is apparent that at a concentration in the neighborhood of 4 to 5 molar, where the solutions contain approximately 10 moles of water per

mole of salt, the character of the conductance process undergoes marked change. At these higher concentrations, the conductance-viscosity product changes but little while the number of water molecules becomes comparable with the number of ions in solution. The hydration of the ions must be changing in this region. Apparently, the increase in mobility due to decreasing hydration just compensates for the increase in viscosity and the conductance-viscosity product remains constant. It will be noted that at the highest concentrations the curves for both salts have a marked upward trend.

Campbell and Kartzmark have measured the conductance and viscosity of concentrated solutions of silver nitrate and ammonium nitrate in water at 95°. Values of the conductance-viscosity product for these salts along with our own for the formates are shown in figure 4, moles of salt per mole of water being plotted as abscissae. At lower concentrations, the product for the two nitrates falls off more sharply than it does for the formates. This is due to the lower values of dielectric constant and viscosity of water at the higher temperature, which results in larger values of the constants α and β of the Onsager equation. At higher concentrations the curves for the two nitrates differ from each other as they do also from those of the formates.

For ammonium nitrate the product decreases continuously up to the highest concentration measured, two moles of water per mole of salt. The product for silver nitrate passes through a pronounced minimum at a concentration which corresponds approximately to 12 moles of water per mole of salt. At concentrations above the minimum, the curve rises and appears to flatten out but its precise form at the higher concentrations cannot be determined because of the scattering of the experimental values.

In going from 22 to 9 moles of water per mole of salt, the conductance-viscosity product for silver nitrate decreases 0.8%; in going from 9 to 6 moles of water per mole of salt the viscosity increases 27% while the conductance-viscosity product increases 9%. To the best of our knowledge, silver nitrate is completely dissociated into its ions. As the number of water molecules per mole of salt decreases, the hydration of the ions must decrease. However, over the concentration range from 22 to 9 moles of water per mole of salt, it is difficult to account for the larger effects that are observed on the basis of hydration alone. One cannot but wonder whether the postulates that underlie the theory of dilute electrolyte solutions remain applicable at high concentrations. The product for ammonium nitrate at higher concentrations is much lower than that of the other salts. One is led to suspect that ammonium nitrate is not completely dissociated into its ions at high concentrations. There is a possibility of hydrogen bonding between the ammonium and the nitrate ions.

From the density data, the volume change accompanying the process of

solution of the two formates at different concentrations has been computed. Values are shown graphically in figure 5. For cesium formate there is a volume contraction of 11.6 cc. at a concentration of 1 molar; it decreases gradually to a value of about 8.5 cc. at 5 molar and remains sensibly constant at higher concentrations. The volume contraction for potassium formate at 1 molar is 8.7 cc.; with increasing concentration the contraction

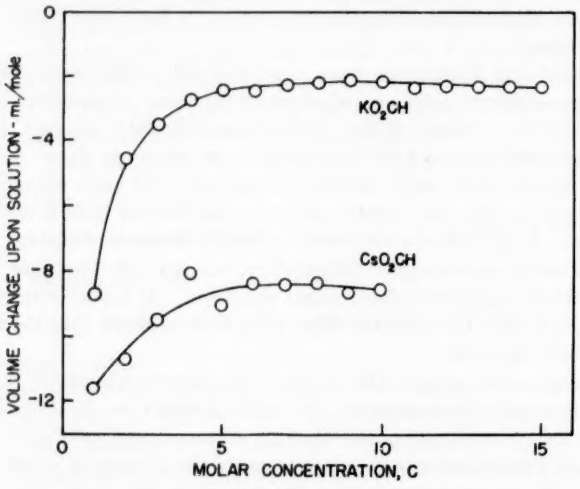

FIGURE 5

decreases sharply and levels off at a value of approximately 2.3 cc. at a concentration of 5 molar. A 5 molar solution contains approximately 8 moles of water per mole of salt. All properties of these solutions indicate a change in the nature of these solutions in the neighborhood of 10 or 8 moles of water per mole of salt. It is difficult to escape the conclusion that the laws governing concentrated salt solutions differ fundamentally from those of dilute solutions. It is not to be expected that the properties of concentrated solutions may be accounted for by merely modifying somewhat the laws that apply at low concentrations.

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† This paper is based on a portion of a thesis presented by M. John Rice, Jr., in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Brown University, June, 1953.

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*DEVELOPMENT AND GENETICS OF A SEX-INFLUENCED TRAIT IN THE LIVERS OF MICE**

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It is generally recognized that there are many traits which may be influenced by the sex of the individual in which they occur. If one of the principal genes necessary for its appearance is located in the Y-chromosome a trait is designated as sex-limited, if in the X-chromosome as sex-linked, and if in addition a major autosomal gene is also necessary the trait is commonly referred to as sex-influenced. However clear-cut these categories may appear in their verbal formulation, it is probably true that one could arrange a finely graded series from traits that are sex-limited in the strictest sense to those whose expression has little or no relation to sex. We wish at this time to report a condition in the mouse liver which might serve as one member of such a series, and to call attention to features in its development which explain why, on genetic grounds alone, this particular trait might sometimes appear to be almost completely sex-limited, and at other times not.

Morphology of the Trait.—The liver of the mouse, like that of many related rodents, is commonly described[1] as consisting of four primary divisions, a right lateral, a medial, a left lateral and a posterior, each of which, except for the left lateral, is in turn subdivided into two major lobes. In the present connection we are concerned with the right lateral division, whose smaller posterio-lateral, and larger anterio-medial components may conveniently be designated as lobes 1 and 2, respectively. The manifestations in which we are especially interested involve only lobe 1, the distinctive features of which may be mentioned briefly.

In the females this lobe has the appearance of a somewhat distorted triangular pyramid whose base is penetrated by the inferior vena cava and whose apex is bent backward over the ventro-medial aspect of the right

kidney. The free part of the lobe presents three well-defined surfaces and three sharp margins, all of which converge to a pointed tip. One surface is in contact with the duodenum, one with the right kidney and suprarenal gland, and one with lobe 2 of the liver and with the peritoneum of the lateral body wall. In some males lobe 1 is similar to that of the female, in others it shows distinct deviations.

When lobe 1 of the male differs from lobe 1 of the female it may do so in any one of several ways. It may show a slight notch in either the duodenal or the renal margin of its anterior surface; the two notches may both be present and connected by a shallow groove; the groove may be deeper causing the apex of the lobe to appear more like an appendage of the basal portion; or, finally, the basal part may itself become pointed and override the morphological tip reducing it to the status of an inferior lobe. There are various minor variations and intergradations, but most specimens fit fairly well into one or another of the six categories set up in table 1, the data for which were derived from some 3500 autopsies on mice representing both sexes and several different strains.

TABLE 1

DISTRIBUTION OF VARIATIONS ACCORDING TO SEX

SEX	LOBE 1 INTACT	DUODENAL NOTCH	RENAL NOTCH	SHALLOW GROOVE	DEEP GROOVE	INFERIOR LOBULE	TOTALS
Males	490	117	170	175	450	492	1894
Females	1598	1	4	1	0	17	1621

Table 1 serves to indicate the distribution of variations in a rather large aggregate, and especially to show differences in incidence between the two sexes. It may be mentioned here that ancestors of some of the mice used had been treated with nitrogen mustard or other possible mutagens, but where no differences were detected between such mice and their controls data from both groups have been included. Excluded from the table are records from earlier autopsies where full details were not recorded, a few doubtful cases, and instances where the configuration of the liver was secondarily modified by partial or complete *situs viscerum inversus*, or by other anomalies. Summarizing the figures, it appears that among these animals deviations from an intact lobe 1 occurred in about 74 per cent of the males and in less than 1.5 per cent of the females. The true difference was possibly slightly greater than this since a few of the variations recorded for females were of a minor or doubtful grade.

When the same figures are broken down according to strain, differences among the males are shown to be considerable, those among the females relatively insignificant. Of five groups in which more than 100 individuals of each sex were examined, the incidence of intact lobes ranged from 97.5 to 100 per cent for the females, and from less than one to over 71 per cent for the males. S-br mice gave the lowest and C3H the highest rates in both

sexes. Figures for the four genetically best defined of these groups are presented in table 2, from which the almost completely consistent data from females have been omitted. The C3H and C57-black animals entered in the table were of the standard strains propagated under these designations by the Roscoe B. Jackson Memorial Laboratory and were derived, respectively, from more than twenty and more than thirty successive generations of brother-sister matings, followed by a further period of close inbreeding in which, however, only part of the matings were between actual sibs. The S-J animals came from a somewhat inbred but rather heterogeneous "general-purpose" stock, and the S-br from a fairly homogeneous strain which has been maintained by the Department of Anatomy at Stanford for a number of years, its most distinctive characteristics being brown fur and a moderately short tail.

TABLE 2

DISTRIBUTION OF VARIATIONS ACCORDING TO STRAIN (MALES ONLY)

STRAIN	LOBE 1 INTACT	DUODENAL NOTCH	RENAL NOTCH	SHALLOW GROOVE	DEEP GROOVE	INFERIOR LOBULE	TOTALS
C3H	82	1	11	2	13	6	115
S-J	145	27	78	81	69	77	477
C57-black	68	22	30	22	108	55	305
S-br	2	11	9	11	125	154	312

TABLE 3

DIRECT GENETIC TESTS (MALE YOUNG ONLY)

NO.	MATINGS: FEMALES	MATINGS: MALES	LOBE 1 INTACT	DUODENAL NOTCH	RENAL NOTCH	SHALLOW GROOVE	DEEP GROOVE	INFERIOR LOBULE	TOTALS
1.	Swiss (4)[a]	Swiss	19	3	3	0	2	9	36
2.	S-br (8, 12)	S-br (11)	0	0	0	0	5	8	13
3.	S-br	Swiss	2	0	2	1	2	0	7
4.	Swiss (1)	S-br	1	2	3	9	20	5	40
5.	F_2 and later		8	5	2	3	11	9	38
6.	S-J (7)	S-J	41	9	4	2	2	2	60
7.	S-J (6)	S-br	0	1	1	0	2	3	7
8.	S-br (2, 12)	S-J	0	3	1	2	7	5	18
9.	F_2 and later		4	1	1	2	11	8	27
10.	C3H (11)	C3H (12)	15	0	5	1	2	0	23
11.	C3H (10)	S-br (2)	0	0	0	1	8	10	19
12.	S-br (2, 8)	C3H (10)	0	5	3	4	18	1	31

[a] Figures in parentheses indicate other matings in which the same mice were involved.

Genetics of Variations in Lobe 1.—Referring to table 2, where only males are listed, it appears that when a distinction is made between intact lobes and those that are more or less subdivided, the former show frequencies of about 71, 30, 21, and 0.06 per cent in the C3H, S-J, C57-black, and S-br males examined in the present series. Corresponding figures for the fe-

males are 100, 98, 99, and 97.5 per cent. Since all the mice were raised in the same laboratory and under similar conditions, these distributions indicate at least a partially genetic background for the observed variations.

Breeding tests were carried out by making the matings first and determining the characteristics of parents and offspring later. About 600 young were obtained from appropriate matings of this sort. Since none of the females in this series showed any subdivision of lobe 1, only males are listed in table 3, which summarizes the results from direct genetic tests. Entries under mating 1 are of indifferent value for present purposes since the records failed in part to differentiate between offspring of males with and those without intact lobes. The items are included only for comparison with those from matings 3 and 4. In all matings except 1 the exact condition of each parent is known. All females involved had an intact lobe 1, all S-br males a deep groove or inferior lobule, all S-J males an intact lobe 1, and the single male used in matings 10 and 12 had a renal notch.

Matings 3 and 4 are the only ones in the series where any of the immediate offspring of the S-br parent of either sex showed an intact lobe 1. Inbred descendants of these matings showed the expected wide distribution of the possible manifestations. Combining figures from the S-J and C3H lines, which seem to be the most similar both genetically and morphologically, we find that when inbred these two strains (matings 6 and 10) produced a total of 83 male young of which 56 had an intact lobe 1, whereas when outcrossed to S-br (matings 7, 8, 11, 12), there were 75 male young none of which had an intact lobe 1. Reciprocal crosses showed no significant differences in distribution of the traits, which indicates that the tendency to a subdivision of lobe 1 is transmitted about equally by both sexes, even though its expression is limited almost exclusively to males.

As to the various grades of lobulation, it may be observed that a vertical line drawn through table 3 between the columns headed "renal notch" and "shallow groove" would divide each group asymmetrically, with the larger fraction either to the right or left of the line. This might suggest a natural separation at this point between two alternative but somewhat overlapping traits. In this connection it may be noted that the C3H male used in mating 10 had a renal notch but behaved in the tests like S-J or other C3H males with intact lobes. The data, however, are not sufficient to differentiate on genetic grounds between the possibilities that there are two alternative traits with incomplete dominance and variable expressivity, or that the several manifestations are individually dependent on varying combinations of multiple or cumulative genes.

Embryology of Variations in Lobe 1.—The ontogeny of lobe 1 throws an entirely new light on the problem and provides a complete change of focus. It appears from a study of embryos in progressive stages of development

that the variations in lobe 1 which have been described are the direct, and not indirect, morphogenic effects of the gonads themselves. In preparing the smaller embryos for study our procedure has been to remove each one from its membranes, immerse it in water for about an hour and then in Bouin's solution for from 6 to 20 hours, depending on its size, after which it was washed in weak alcohol and dissected under the low power of a binocular microscope. In these dissections the routine first step was to remove the skin and subjacent tissues from the right side of the back down to the level of the kidney, duodenum, and liver.

In embryos at about 12 to 13 days after fertilization, and of approximately 10 mm. in length,[2, 3] lobe 1 is the most rudimentary of the seven major portions of the liver. At this stage it is very small and compressed on three sides by the much larger lobe 2, the right kidney and the duodenum, while the relatively undifferentiated right gonad and its accompanying ducts cover it and project into its dorsal surface. This gives the lobe as viewed from above somewhat the appearance of a boat or dugout, with a central depression and two raised margins embracing the ventral portion of the gonad. Development of embryos from this point on is rapid,[4–6] and this is especially true of the region under consideration. At about this time the ovary, while still small and cylindrical, begins to move obliquely across the dorso-caudal pole of the kidney, early freeing lobe 1 in the female to undergo a considerable dorsal expansion, in the course of which the groove that had been occupied by the gonad generally becomes completely smoothed out leaving no trace of its former existence. The male gonad, by contrast enlarges considerably before beginning its descent, and this has the effect of still further deepening and widening the groove as well as somewhat retarding the development of lobe 1 as a whole. When the testis finally does descend, it is not immediately lifted off lobe 1, as is the ovary, but continues to impinge on its dorsal surface until it has completely passed over the duodenum and on into the pelvis. By the time the testis has lost contact with lobe 1, the two sides of the latter have often developed appreciably, accentuating the depression between them and causing it to remain as a permanent notch or groove.

Differential early development on the two sides of the groove apparently determines the precise configuration which the lobe will ultimately assume, and all forms of adult lobulation may be found taking shape in embryos of around 15 to 16 days. That in some adult male livers lobe 1 shows these traits and in others does not is probably due to the length of time during which the mechanical impact of the testis persists. That this period varies is demonstrated by the fact that sometimes in members of the same litter one right testis will be found deep in the groove, another just leaving it, and a third in the pelvis well beyond the liver. It is, of course, impossible to follow individual development in preserved specimens, but exami-

nation of a considerable mass of embryonic material makes it seem very probable that those rudiments which are early freed from the impeding influence of the testis are the ones which develop as intact lobes, while those in which the testis remains until the liver has bulged out around it are the ones which in the adult show varying degrees of lobulation. It would thus appear that the immediate factor determining whether or not lobe 1 will be intact is a temporal one, involving the time and speed at which the testis loses contact with the liver.

Discussion.—It has seemed worth while to record these rather trivial variations in the mouse liver because of the bearing they may have on questions of wider interest. In the first place, it is instructive to find that a trait which in one strain might appear to be almost completely sex-limited can be shown in another strain not to be so in any strict genetic sense. In this instance, the genes immediately involved are clearly autosomal and probably effective through their regulation of developmental rate at a crucial moment in embryonic life. For their effect to be made apparent at all, however, there must be the added impediment of a gonad, the presence of which in its turn is determined by sex chromosomes. Existence of the autosomal part of the complex would probably never be suspected from the examination of female material even though its manifestations are clearly recognizable in the male.

It is probably significant that more than thirty generations of intensive inbreeding in the C57-black line has failed to fix a trait that is obviously hereditary (albeit with varying penetrance and expressivity), while less intensive selection has come much closer to doing so in the S-br line. The latter has been maintained for some ten years with no special breeding program other than the periodic reduction of the stock to three or four selected individuals. One might wonder if some strains of mice which persist with fair vitality despite intensive inbreeding are in reality being continually selected for vigor which fortuitously happens to be associated with heterozygosity in certain unsuspected genes, as professor P. W. Gregory[7] believes to be the case in some herds of cattle.

On the embryological side, if the inferences drawn here are substantiated by such experimental tests as it would seem feasible to make, the development of the lobe 1 in the mouse liver will afford one more illustration of the importance of "developmental rate in structural expression" often emphasized by Professor Stockard[8] and later embryologists.

Summary.—Certain lobulations or incisions in the more caudal lobe (lobe 1) of the right lateral division of the mouse liver are found to occur with a rather high frequency in males, only rarely in females. A genetic and embryological study of these manifestations reveals that they are the result of impressions made by the gonads in early embryonic life, and that differences in grade of expression are apparently mediated through auto-

somal genes rather than those in the sex chromosomes. The difference in incidence between the sexes is due to the fact that the ovary is early removed from contact with the liver, while the testis commonly maintains such contact with resulting pressure on lobe 1 during the period of its most rapid development. The evidence indicates that grades of expression, which in the male range from slight to marked deviations from the female type, are conditioned by the time at which the gonad in its descent loses contact with the liver, the final expression being in effect an index of the developmental rate at a period around the fifteenth or sixteenth day of intra-uterine life.

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MUTATION RATES AND LETHAL GENE FREQUENCIES IN POPULATIONS OF DROSOPHILA MELANOGASTER

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It is now generally accepted that gene mutation is the basis of evolutionary genetic change. Though many data are available concerning the relative frequencies of mutations in populations of several species of Drosophila,[1] there is a paucity of information regarding spontaneous mutation rates in natural populations of these species. The present article is a report of preliminary investigations of sex-linked lethal mutation rates in natural and artificial cage populations of *D. melanogaster* and the frequency of second chromosome lethals in the artificial populations.

The Experimental Procedure.—The samples of natural populations analyzed were obtained from Blacksburg, Va.; Canonsburg, Pa.; Austin, Tex.; Chapel Hill, N. C.; and Amherst, Mass.

All samples, with the exception of Chapel Hill, were handled in the fol-

lowing manner. As soon as the flies were obtained from the field, from 40 to 100 females were isolated singly in 25 × 95-mm. vials of a standard culture medium and placed at 25°C. One F_1 male, no more than three days old, was taken from each of the cultures. He was mated for two days to two females carrying the X-chromosome complex known as Muller-5. The females were allowed to oviposit for a week. The F_2 were shaken in each case to a new culture where they remained for two days to insure maximum insemmination. Then they were anesthetized and put into individual cultures, one pair per vial, for the standard Muller-5 sex-linked mutation rate test. Each family of test cultures from a single F_1 male was numbered. There were about fifty tests in most of the families, meaning tests of 50 sperm for the F_1 male. The numbering of each family is essential since the occurrence of more than one mutation within a family might result from a single mutation in the F_1 male early in spermatogenesis, thus affecting more than one sperm. When two or more mutations occurred within a family, they were localized by cross-over tests with the X-chromosome markers, yellow, cut, raspberry, forked (*y ct ras f*) in order to ascertain how many independent mutations they represented.

The test generation was scored for lethal mutations by means of a binocular microscope. The presence of three or more normal males together in the field was considered sufficient to score the test as non-mutant. The field showed a maximum of about thirty flies. All other cases were anesthetized for scoring. In cases of less than 10 per cent normal males, retest matings were made, and the final scoring was based on a minimum count of 100 flies in two generations. Tests showing no normal males were scored as lethals. Those showing up to 10 per cent normal males were scored as semilethals. Obvious phenotypic mutations were also scored. The mutation rate was based on the total of the three kinds of mutations which occurred independently in each family. All lethals from the same family which appeared to be at the same locus in cross-over tests were considered to be the result of a single mutation. Such cases were rare in the study.

Only a small number of females were available from Chapel Hill. They were allowed to lay eggs in half-pint bottle cultures, from which many young F_1 males were mated individually to Muller-5 females as above.

The sex-linked lethal mutation rate is being followed in two cage populations of *D. melanogaster*. The cages used and the technique of their maintenance are described by Wallace.[2] The first population, known as *M*, was derived from a collection of flies from South Amherst, Mass., in September of 1952. Fifty fertilized females, sisters of the males tested in the Amherst sample, were used to start the cage on October 3, 1952. The cage was kept at 25°C. Egg samples were removed from the cage at approximately monthly intervals from the time of the emergence of the

first generation in the cage. Resulting young males were mated singly to Muller-5 females and tested as above.

The second cage population, known as *O*, was derived from an approximately homozygous Oregon-R strain which had been inbred for 95 generations by brother-sister matings; the last 40 generations in this laboratory. The 50 females which were used to start the cage and their brothers which were used for an initial determination of mutation rate were obtained from a half-pint laboratory stock culture. The cage population began on November 17, 1952, and was treated in the same manner as the *M* population. The two cages were kept in the same incubator.

In order to determine the second chromosome lethal gene frequencies in the *M* and *O* populations, adult males were removed at approximately

TABLE 1

MUTATION RATES IN NATURAL POPULATIONS

LOCALITIES	COLLECTION TIME	NO. OF TESTS	NO. OF LETHALS	NO. OF SEMI-LETHALS	MUTATION RATE
Blacksburg (Va.)					
1	May '52	3768	3	0	0.80×10^{-3}
2	Oct. '52	2550	3	0	1.18×10^{-3}
Canonsburg (Pa.)	July '52	2571	6	1	2.72×10^{-3}
Austin (Tex.)	Nov. '52	3323	6	0	1.81×10^{-3}
Chapel Hill (N. C.)	Nov. '52	3456	5	1	1.14×10^{-3}
Amherst (Mass.)	Sept. '52	3191	3	2	1.56×10^{-3}

TABLE 2

MUTATION RATES IN THE *M* (AMHERST) CAGE

SAMPLE	TIME (DAYS FROM START OF CAGE)	NO. OF TESTS	NO. OF LETHALS	NO. OF SEMI-LETHALS	MUTATION RATE
1	0	3191	3	2	1.56×10^{-3}
2	33	3326	7	3	3.01×10^{-3}
3	67	3829	12	1	3.40×10^{-3}
4	98	3310	9	0	2.42×10^{-3}

monthly intervals and the lethal frequency was determined in the manner described by Ives.[3] Lethals and semilethals (those yielding less than 17 per cent normal flies) have been grouped together in the tables.

Mutation Rates.—The mutation rates determined for the five natural populations studied are given in table 1. The variability in the rates from population to population is not significant. (P is 0.47 as determined by chi-square.) The populations studied represent collections of flies from geographically diverse habitats, but it is by no means certain that the larval habitat of the populations differed significantly in an ecological sense during the time of greatest reproductive activity.

Recorded in table 2 are the mutation rates measured for the *M* popula-

tion. Sample 1 is the mutation rate at the time the cage was established. The remaining values were obtained from egg samples taken from the cage at the times indicated in the table. A P value of 0.70 for samples 2, 3, and 4 indicates only random variation. However, upon comparing the pooled data of the M population with the pooled data of the natural populations one obtains a P value of 0.016 indicating a significant difference between the M cage population and the natural populations with respect to mutation rate.

Table 3 shows the mutation rates of the O population. Sample 1 is

TABLE 3

MUTATION RATES IN THE O (OREGON-R) CAGE

SAMPLE	TIME (DAYS FROM START OF CAGE)	NO. OF TESTS	NO. OF LETHALS	NO. OF SEMI-LETHALS	MUTATION RATE
1	0	3278	2	2	1.22×10^{-3}
2	33	3412	5	0	1.47×10^{-3}
3	63	2835	5	0	1.76×10^{-3}
4	97	3112	7	0	2.25×10^{-3}

TABLE 4

SECOND CHROMOSOME LETHAL GENE FREQUENCIES

SAMPLE	TIME (DAYS FROM START OF CAGE)	TESTS	NO. OF LETHALS	PER CENT OF LETHALS
		M Population		
1	0	190	72	37.9
2	19	105	24	22.9
3	49	200	60	30.0
4	81	133	35	26.3
5	112	125	36	28.8
6	152	55	19	34.5
		O Population		
1	0	231	3	1.3
2	22	205	3	1.5
3	53	161	4	2.5
4	84	162	7	4.3

the mutation rate of the inbred Oregon-R stock at the time that the cage population was established. A P value of 0.85 for samples 2, 3, and 4 suggests only random variation. Since the past history and the artificial condition of the O population is quite different from that of the natural populations, there is nothing instructive in a comparison of the mutation rates of the O population and the five natural populations. However, the mutation rates of samples 2, 3, and 4 from both the M and O populations make a valuable comparison between flies with different past histories subjected to similar cage environments. Such a comparison by a chi-

square test gives a P value of 0.26. The differences between the two populations are not significant, due to the almost linear increase in the mutation rate in the O population to a point very close to the mutation rate of the M population in the last sample.

Lethal Gene Frequencies.—The second chromosome lethal gene frequencies determined for both the M and O populations are given in table 4. It will be noted that the M population had an initial chromosome lethal gene frequency of 37.9 per cent. This represents the lethal frequency in the sample of the Amherst natural population from which F_1 flies were used to establish the cage population. Subsequent samples showed a decrease in frequency in the first generation in the cage (sample 2) and what appear to be random fluctuations thereafter.

The O population, which at its inception had a lethal gene frequency of 1.3 per cent, has shown a steady increase in frequency in subsequent samplings.

Discussion.—It has been claimed by Berg[4, 5] that sex-linked lethal mutation rates in different natural populations of *D. melanogaster* vary from population to population according to ecological differences. She offers the hypothesis that the mutation rate is subject to natural selection and that rates are lower in populations existing under specialized ecological situations in which the range of adaptedness of the individual is narrow. On the other hand, populations found in more generalized environments and thus under conditions in which there would be wide range of adaptedness would be expected to have a higher mutation rate. The action of natural selection would not be expected to be as stringent here when compared to the situation existing in the specialized environments. Though her data are not suggestive, they are based on a relatively small number of tests and experimental procedures are often obscure. However, the results of Dobzhansky, Spassky, and Spassky[6] obtained from an analysis of lethal mutation rates in *D. willistoni* and *D. prosaltans* are of interest in this respect. The former species is common throughout most of tropical America while the latter is a rare species, restricted to a small number of localities. It has been shown that the mutation rate is significantly higher in the widely distributed *D. willistoni* when compared with *D. prosaltans* which appears to require specialized environments for its maintenance. Thus, their results will tend to support the Berg hypothesis. They point out further that, "if mutation rates are adaptively modified by ecological conditions, the divergence among species may be expected to be greater than among populations of a single species." The results presented here for a number of possibly ecologically diverse populations of *D. melanogaster* bear this out. However, the mutation rates in artificial populations present a somewhat different picture. Here, as was the case with the M population, the mutation rate increased significantly above the character-

istic for the natural populations. In addition, the *O* population appears to exhibit a steady increase in mutation rate above that found to be characteristic of the inbred Oregon-R stock.

These results suggest that mutation rate can be modified in populations permitted to develop under completely new environmental conditions. The representatives of the Amherst population in the population cage are subjected to an entirely new spectrum of selective agents; competition is keen for food as well as for egg laying and pupation sites. It is possible, therefore, to conceive of the increased mutation rate as being the consequence of a population's response to natural selection under the new conditions afforded by the cage. This would fit at least in part, the hypothesis of Berg. The drop in the second chromosome lethal gene frequency the initial 37.9 to 22.9 per cent after the first cage generation had developed was not, however, due to cage conditions. It was due, in all probability, to elimination of lethal chromosomes caused by the vigorous brother-sister mating of flies which were put into the cage at the start.

The *O* population showed a steady increase in mutation rate. The ancestors of the cage population have been subjected to the rather specialized conditions of laboratory culture for 95 generations. Genetic variability was kept at a minimum level by inbreeding and any factors tending to increase mutation rate would also be kept at a minimum mutation rate level by this process. However, when permitted to outcross in the population cage, the lethal gene frequency begins to rise and undoubtedly other genetic variability also increased. Similar increases in lethal gene frequency have been obtained for a number of cage populations by Wallace.[7] Accompanying the increase in lethal frequency is an apparent gradual rise in mutation rate. This increase can be explained either on the basis of a relaxation of selection following the Berg hypothesis or by the action of mutator gene complexes developing under cage conditions of increased population size. Thus, the increase in mutation rate is perhaps a reflection of the greater diversity of the cage environment as compared with the conditions obtaining in single vials of brother-sister single pair matings.

The findings of no significant differences between mutation rates of samples of flies taken from different populations of *D. melanogaster* is not in exact accord with the finding of Demerec.[8] However, most of his samples showed a low mutation rate and only a few were high. In addition, his samples were derived from stocks which had been kept under laboratory conditions for several generations. It may be that tests of additional areas, using our technique, will show some to be significantly different. None of the wild samples came from areas of really small breeding units as indicated by their frequency of second chromosome lethals (data to be published by Ives).

It is possible that the apparent difference in mutation between *D. prosaltans* and *D. willistoni* is due as much to differences in mutator alleles

in populations of different sizes as it is to ecological differences between their habitats. Having a much larger population area and a larger breeding population size, *D. willistoni* would be expected to have a higher concentration of mutator alleles similar to the mutator *high* described by Ives[9] and hence a higher mutation rate than *D. prosaltans*. This is what was observed to be the case. However, the difference in mutator complexes and in population size would both depend in turn on the different ecological requirements of the two species. They may, therefore, be mechanisms by which ecological differences can express themselves in the mutation rate.

Further light on the nature of the changes in the frequency of lethals in the *M* cage should come from allelism tests of the lethals recovered in the various samples. Since only 200 second chromosomes, at the most, constituted the gene pool, there should be a higher amount of allelism in samples of lethals taken from the population cage than in the initial sample from the much larger natural population. Preliminary results indicate that this expectation will be realized.

It is obvious that much more extensive work needs to be done to elucidate the problems arising in this preliminary study of the dynamics of natural and artificial populations of *D. melanogaster*. In summary, it may be stated that preliminary studies of mutation rates in flies derived from five geographically diverse natural populations of *D. melanogaster* showed a comparative uniformity. An artificial population derived from flies collected in one area and maintained in a cage showed an increase in mutation rate over that found in natural populations. A cage population developed from a stock with a long history of close inbreeding also showed a tendency for increased mutation rate. It is suggested that these mutation rate increases reflect the effects of the cage environment.

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ANTI-f AND THE "NEW" Rh ANTIGEN IT DEFINES

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A "new" *Rh* antibody, anti-*f*, has been found in the serum of a man, Mr. Cl., aged 30 who suffers from hemophilia. A brief summary of the investigation of this antibody has recently been published.[1]

Since childhood Mr. Cl. has received about 35 transfusions of blood, the last of which, in December, 1951, was followed by a rigor. He was referred to us, for the first time, in March, 1952, by the Hemophilia Foundation, Inc. At that time we found three blood-group antibodies in his serum: anti-*B*, anti-*K*, and anti-*S*. In addition there was at least one other unidentified antibody. In the hope of clarifying the reactions of this antibody, 0.25 ml. of *O, NsNs, cdE/cde, kk* blood was injected on two occasions: following this the presence of anti-*N* was disclosed and, in addition, the "new" antibody, anti-*f*.

The blood groups of Mr. Cl. are: A_1, *MsMs*, *P+*, *CDe/cDE*(R_1R_2), *Lu*($a-$), *kk*, *Le*($a+b-$), *Fy*($a+$), *Jk*($a+b+$). His serum can be absorbed free of anti-*B*, anti-*N*, anti-*S*, and anti-*K* leaving anti-*f* alone; it is the anti-*f* left in this absorbed serum that is the subject of this paper. A detailed account of the *Rh* blood groups prior to the recognition of anti-*f* may be found in the text of Race and Sanger.[2]

THE REACTIONS OF ANTI-*f*.

Methods.—The anti-*f* in the serum of Mr. Cl. was found to react most clearly with an albumin technique: a volume (0.01 ml.) of a 4% suspension of red cells in 20% bovine albumin was added to an equal volume of the serum. The mixtures were left at 20°C. for from one to two hours, then centrifuged at about 1,000 r. p. m. for 90 seconds. The cell deposit was then pipetted on to a slide; if agglutination was not visible to the naked eye it was looked for with the low power of the microscope.

More than half of the red cell samples were tested against titrations of anti-*f*, the diluent being 20% albumin; the rest were tested against the undiluted serum. The dilutions were made in bulk and then distributed to the appropriate tubes.

Scores were given to the reactions: agglutination visible to the naked eye scored 10, lesser positive reactions scored 8, 5, 3, or 2. It soon became clear that there was a marked dosage difference, for *cde/cde, cDe/cde* and *cD^{u}e/cde* score more highly than do other positive samples:

65 *cde/cde*, *cDe/cde* and *cD^{u}e/cde* samples; mean score = 32.5

99 other positive samples; mean score = 16.0

An indication of the variability of the method is given by the following scores for a *cde/cde* person (J.E.N.) tested on 12 occasions spread over 5 months.

35, 44, 30, 30, 33, 36, 38, 35, 35, 38, 33, 34; mean = 35.1 ± 3.8

and for a *CDe/cde* person (M.E.M.) tested on 11 occasions:

6, 5, 18, 12, 10, 11, 13, 12, 7, 10, 5; mean = 9.9 ± 4.0

Results: The Usual Reactions.—Table 1 shows the results of testing with anti-*f* samples of blood from 328 unrelated white persons and from 125 of their relatives. From these results it is clear that the antigen recognized by anti-*f* is part of the *Rh* complex of antigens; this is also shown by the linkage tests described below. Contingency tables show that there is no evidence of an association between the *f* antigen and any other of the known blood-group antigens, sex, or the ability to taste phenyl thiocarbamide.

Many of the "unrelated" samples in table 1 were selected, as can be seen from the rarity of their *Rh* groups. It can be calculated that anti-*f* will agglutinate approximately 66% of samples of blood from unselected white people.

The reactions of anti-*f* have been confirmed by absorption tests with red cells representing most of the critical genotypes. A volume of serum, 0.3 ml., was absorbed with an equal volume of packed cells for 15 minutes, at 20°C.; four successive absorptions were done. The following red cells failed to absorb anti-*f*: *CDe/CDe* (6 samples), *cDE/cDE*, *CDe/cDE*, *Cde/cdE* (2 samples). The following red cells did absorb anti-*f*: *cde/cde*, *cDe/cde*, *CDe/cde* (2 samples), *cDE/cde*, *cdE/cde*, *Cde/cde*, *CDE/cDe*, and *CdE/cde*; as expected *cde/cde* and *cDe/cde* were the most efficient bloods for absorption. Anti-*f* could be eluted from the various absorbing cells and the eluates when tested behaved alike, thus giving direct evidence that the positive reactions of the anti-*f* serum with red cells of different genotypes are due to one antibody.

Table 1 becomes clear when it is realized that anti-*f* is reacting with a product of the chromosomes *cde*, *cDe*, and *cD^{u}e*. These chromosomes may consequently be distinguished by the addition of *f*. If the absence of *f* is indicated by *F* the chromosomes are:

cdef, *cDef*, *cD^{u}ef*,

CDeF, *cDEF*, *C^{u}DeF*, *CdeF*, *cdEF*, *CD^{u}eF*, *cD^{u}EF*,

CDEF, *CD^{u}EF*, *C^{u}deF*, *CdEF*, *cDE^{u}F*, *C^{u}DeF*, and *C^{u}dEF*.

This shows the usual arrangement of *f* in Caucasian chromosomes, and the evidence is given in table 2.

Less Common Reactions.—For reasons given below we expect certain less common combinations to exist—namely *cDeF, cdeF, CDef* and *cDEf.*

TABLE 1

THE REACTIONS OF ANTI-*f* WITH THE RED CELLS OF 328 UNRELATED, BUT OFTEN SELECTED, WHITE PEOPLE AND 125 OF THEIR RELATIVES

GENOTYPE	POSITIVE		NEGATIVE	
	UNRELATED	RELATIVES	UNRELATED	RELATIVES
CDe/cde	84	26	0	0
cde/cde	61	28	0	0
cDE/cde	30	18	0	0
cDe/cde	19	4	0	0
cdE/cde	9	0	0	0
Cde/cde	4	2	0	0
C^{w}De/cde	1	0	0	0
CD^{u}e/cde	4	1	0	0
CDe/cDe	1	1	0	0
cDE/cDe	0	1	0	0
CdE/cde	1	2	0	0
C^{w}de/cde	1	0	0	0
CDE/cDe	1	0	0	0
CDE/cde	0	1	0	0
CD^{u}E/cde	1	2	0	0
cD^{u}e/cde	3	0	0	0
C^{u}De/cde	1	0	0	0
C^{w}dE/cde	1	2	0	0
CDe/CDe	0	0	52	19
CDe/cDE	0	0	25	10
cDE/cDE	0	0	12	0
C^{w}De/CDe	0	0	5	0
C^{w}De/cDE	0	0	2	0
CDe/Cde	0	0	2	0
cDE/cdE	0	0	1	3
CDe/cdE	0	0	1	1
cDE/Cde	0	0	0	1
CDe/CDE	0	0	1	0
Cde/cdE	0	0	2	0
CDe/CdE	0	0	0	1
CDe/CD^{u}e	0	0	1	0
cD^{u}e/cdE	0	0	0	1
Cde/Cde	0	0	1	0
cDE^u/cde	0	0	0	1
−D−/−D−	0	0	1	0

We have found an example of *cdeF/cdef.* The red cells of a member of the Staff of the Lister Institute, although *cde/cde,* persisted in giving a single dose score with anti-*f:* his scores were 9, 13, 8, 10, 12, and 7 as against an average for all other *cde/cde* of 32.5 (the next lowest score for any

unrelated *cde/cde* was 21). The absence of *f* from one of his *cde* chromosomes is confirmed by family studies. His sister is also *cdeF/cdef* for she scores only 14. Their father is *cDE^{u}F/cdeF* since his red cells are not agglutinated by anti-*f* nor will they absorb it (family 19, table 3).

TABLE 2

EVIDENCE FOR THE EXISTENCE OF THE FOLLOWING CHROMOSOMES

CHROMOSOMES	BLOOD SAMPLES NEGATIVE WITH ANTI-*f*	SAMPLES POSITIVE WITH ANTI-*f*	
		SINGLE DOSE	DOUBLE DOSE
cdef		*CDe/cde, cDE/cde* *Cde/cde, cdE/cde* *C^{u}De/cde, CdE/cde* *CD^{u}e/cde, CD^{u}E/cde* *C^{u}de/cde, CDE/cde*	*cde/cde* *cDe/cde* *cD^{u}e/cde*
cDef		*CDe/cDe, cDE/cDe* *CDE/cDe*	*cDe/cde*
cD^{u}ef			*cD^{u}e/cde*
CDeF	*CDe/CDe, CDe/cDE, C^{u}De/CDe* *CDe/CdE, CDe/CDE, CDe/Cde* *CDe/cdE, CDe/CD^{u}e*	*CDe/cde, CDe/cDe*	
cDEF	*cDE/cDE, CDe/cDE, C^{u}De/cDE* *cDE/Cde, cDE/cdE*	*cDE/cde, cDE/cDe*	
cdEF	*CDe/cdE, cDE/cdE, Cde/cdE* *cD^{u}E/cdE*	*cdE/cde*	
CdeF	*CDe/Cde, cDE/Cde, Cde/cdE* *Cde/Cde*	*Cde/cde*	
C^{u}DeF	*C^{u}De/CDe, C^{u}De/cDE*	*C^{u}De/cde*	
CDEF	*CDe/CDE*	*CDE/cDe, CDE/cde*	
CdEF	*CDe/CdE*	*CdE/cde*	
C^{u}dEF		*C^{u}dE/cde*	
C^{u}deF		*C^{u}de/cde*	
CD^{u}eF	*CDe/CD^{u}e*	*CD^{u}e/cde*	
cD^{u}EF	*cD^u/cdE*		
CD^{u}EF		*CD^{u}E/cde*	
C^{u}DeF		*C^{u}De/cde*	
cDE^{u}F	*cDE^u/cde**		
−D−−†	*−D−/−D−*		

* The genotype is *cDE^{u}F/cdeF*, see text.

† The chromosome *−D−* does not carry *f*. Until anti-*F* is found there must be some doubt whether it is *−D−−* or *−D−F*.

We thought we had identified the chromosome *cDeF* when a sample of blood apparently *cDe/cde* persisted in giving a single dose score with anti-*f*: ultimately we found that this sample was in fact *C^{u}De/cde* for the cells were agglutinated by some anti-*C* sera but not by others.

Family investigations.—Table 3 shows the results of testing 47 families.

TABLE 3

THE RESULTS OF TESTING 47 FAMILIES, OR PARTS THEREOF, WITH ANTI-*f*

FAMILY NO.	FATHER	MOTHER	CHILDREN					
1	− *CDe/CDe*	− *CDe/cDE*	− *CDe/CDe*	− *CDe/CDe*				
2	− *CDe/CDe*	− *CDe/CDe*	− *CDe/CDe*	− *CDe/CDe*				
3	− *CDe/CDe*	− *CDe/CDe*	− *CDe/CDe*	− *CDe/CDe*	− *CDe/CDe*	− *CDe/CDe*		
4	− *cDE/Cde*	*cDE/cdE*	− *Cde/cdE*					
5	− *CDe/cdE*	*cD^{u}E/cde*	− *cD^{u}E/cdE*	*cdE/cde*				
6	*CDe/cDE*	*Cde/cde*	− *CDe/Cde*					
7	+ *cDE/cde*	− *CDe/Cde*	+ *Cde/cde*	− *CDe/cDE* or *Cde/cDE*	+ *Cde/cde*			
8	− *CDe/cDE*	+ *cdE/cde*	− *cDE/cdE*	− *cDE/cdE*				
9	− *CDe/CD^{u}e*	+ *cDE/cde*	+ *CDe/cde*	+ *CD^{u}e/cde*	+ *CDe/cde*	− *CDe/cDE*		
10		− *CDe/cDE*	+ *cDE/cde*	+ *CDe/cde*	+ *CDe/cde*	− *CDe/cDE*		
11	+ *cDE/cde*	− *CDe/cDE*	− *CDe/cDE*	− *cDE/cDE*				
12	+ *CDe/cde*	− *CDe/cDE*	+ *cDE/cde*	+ *cDE/cde*	− *CDe/cDE*	− *CDe/CDe*		
13	− *CDe/CDe*	+ *cdE/cde*	+ *CDe/cde*	− *CDe/cdE*				
14	+ *CDe/cde*	− *CDe/CDe*	+ *CDe/cde*	− *CDe/CDe*				
15	− *cDE/cDE*	+ *cde/cde*	+ *cDE/cde*	+ *cDE/cde*				
16	− *CDe/CDE*	*cde/cde*	*CDE/cde*	*CDE/cde*				
17	− *Cde/cdE*	*cde/cde*	*Cde/cde*	*Cde/cde*				
18	+ *cDE/cDe*	*cDE/CDE*	+ *CDE/cDe*					
19	− *cDE^{u}/cde*	+ *CDe/cde*	+ *cde/cde*	+ *cde/cde*				
20	+ *CDe/cde*	+ *CDe/cde*	− *CDe/CDe*	+ *cde/cde*				
21	+ *CDe/cde*	+ *CDe/cde*	+ *CDe/cde*	− *CDe/CDe*				
22	+ *cDE/cde*	+ *CDe/cde*	− *CDe/cDE*					
23	+ *CDe/cde*	+ *CDe/cde*	− *CDe/CDe*	− *CDe/CDe*				
24		+ *CDe/cde* or *CDe/cDe*	+ *cDe/cde*	− *CDe/CDe*	− *CDe/CDe*	− *CDe/CDe*	− *CDe/CDe*	

Notes.—The fathers in families 1, 28, and 29 are brothers. The mothers in families 10 and 11 are sisters and so are the mothers in families 15 and 24. The father in family 22 is the first child in family 10, and the father in family 45 is the child in family 18. The mother in family 47 is the third child in family 46.

TABLE 3 (Continued)

FAMILY NO.	FATHER	MOTHER	CHILDREN					
25	cDE/cde	cdE/cde	− cDE/cdE	− cDE/cdE				
26	CDe/cde	+ cDE/cde	− CDe/cDE	+ cde/cde	+ cde/cde			
27	cDE/cde	+ CD^{u}E/cde	+ CD^{u}E/cde	cDE/cde	cde/cde	cDE/CD^{u}E	+ CD^{u}E/cde	
28	+ CDe/cde	+ cde/cde	+ cde/cde	+ cde/cde				
29	+ cDE/cde	+ cde/cde	+ cDE/cde	+ cDE/cde	+ cDE/cde			
30	+ CDe/cde	+ cde/cde	+ cde/cde	+ CDe/cde				
31	+ cDE/cde	+ cDE/cde	+ cDE/cde	+ cDE/cde	+ cDE/cde			
32	+ CDe/cde	+ CDe/cde	+ cde/cde	+ CDe/cde	+ CDe/cde			
33	+ cDE/cde	+ CDe/cde	+ cde/cde	+ CDe/cde	+ cDE/cde			
34	+ CDe/cde	+ cde/cde	+ CDe/cde	+ CDe/cde	+ cde/cde	+ cde/cde	+ CDe/cde	
35	+ CDe/cde or CDe/cDe	+ cDE/cDe or cDE/cde	+ cDe/cde					
36	+ CDe/cDe	+ cde/cde	+ cDe/cde	+ cDe/cde				
37	+ CDe/cde	+ cde/cde	+ cde/cde	+ cde/cde	+ CDe/cde			
38	+ cde/cde	+ CDe/cde	+ cde/cde	+ cde/cde	+ cde/cde	+ CDe/cde	+ CDe/cde	+ cde/cde
39	+ CDe/cde or CDe/cDe	+ CDe/cDe or CDe/cde	cDe/cde					
40	+ cDE/cde	+ cde/cde	+ cDE/cde					
41	+ cde/cde	+ cde/cde	+ cde/cde	+ cde/cde	+ cde/cde	+ cde/cde	+ cde/cde	+ cde/cde
42	+ CD^{u}e/cde	+ cde/cde	+ cde/cde	+ cde/cde				
43	+ cDE/cde	+ cde/cde	+ cDE/cde	+ cde/cde	+ cDE/cde			
44	+ cde/cde	+ CDe/cde	+ cde/cde	+ cde/cde				
45	+ CDE/cDe	+ cde/cde	+ CDE/cde	+ cDe/cde	+ cDe/cde			
46		+ C^{w}dE/cde	CDe/cde	CDe/cde	+ C^{w}dE/cde	CDe/cde		
47		+ C^{w}dE/cde	+ C^{w}dE/cde					

Notes (Continued).—Twins are bracketed, all are monozygous save the second pair in family 12.

The *Rh* groups of the following families have previously been published: 1, 4, 5, 18, 21, 27, 28, 29, 45, 46, and 47. They should not therefore be included in any compilation of genetical studies of the *Rh* groups.

In 11 of the families not all the members have been tested with anti-*f* but their *Rh* groups are given from our records because they reveal the true *Rh* genotype of the members who have been tested.

Without any of the foregoing considerations, association of anti-*f* with the *Rh* system would be apparent from the families. Ten of them, families 7, 8, 9, 10, 11, 12, 13, 14, 23, and 24, are of the type to show linkage of the *f* antigen with the *Rh* locus, and this they do with no example of crossing-over. Applying the *u* statistics of Fisher,[3, 4] as elaborated by Finney,[5] linkage at the 1 in 100 level of probability is established between *f* and *Rh*, for $\Sigma(\lambda)$, which is 36, is greater than $2.33\sqrt{\Sigma(k)}$, which is 18.9.

THE RELATION OF THE *f* ANTIGEN TO THE *Rh* SYSTEM

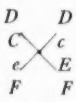
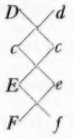
The antigen recognized by anti-*f* clearly belongs to the *Rh* system, but it cannot be identified with any of the known members of the 3 series of allelic antigens: among the genotypes which are negative with anti-*f* (table 1) are to be found all the known *Rh* antigens (save c^v which we have not tested) and, furthermore, all the common antigens are to be found there in the homozygous state, *DD*, *dd*, *CC*, *cc*, *EE*, and *ee*.

TABLE 4

THE CROSS-OVER THEORY OF FISHER EXTENDED TO INCLUDE *f* AND *F*

The Common Chromosomes Are Known to Be *CDeF*, *cdef*, and *cDEF*: the Table Shows the Expected Chromosomes of the Second Order of Frequency.

COMMON HETEROZYGOTES WITH THEIR APPROXIMATE FREQUENCIES

33%	12%	11%
		

Recombinations after a Single Cross-Over

dCeF	*DCEF*	*dcEF*
Dcef	*DceF*	*Dcef*
dceF		*dceF*
DCef		*DcEf*

A fourth allelic series of antigens.—Anti-*f*, and the antigen it defines, will easily fit in with what is already known of the *Rh* system only if we postulate a fourth series of allelic antigens. The antigen *f* shows preferences for its partner antigens: it prefers *cde*, *cDe*, and cD^ue, though it may desert at least *cde*. This is typical of the behavior of the other *Rh* antigens. The crossing-over idea of Fisher, which takes into account these preferences, not only accommodates *f* but also gains support from it.

Table 4 shows the crossing-over idea extended to include *f* and its pre-

sumptive allele, *F*. The right order appears to be *DCEF*. The order *FDCE* would require all *cDe* chromosomes to lack *f*, the order *DFCE* would require the majority of *cDe* to lack *f* and the order *DCFE* would require a high proportion of *cDe* to lack *f:* in fact, all *cDe* chromosomes so far tested have *f*.

Thus having allowed the chromosome *cDe* to dictate the position of *F* in relation to *DCE*, certain consequences follow, which may be seen from table 4. *Cde* should lack *f*, which it does; *cdE* should lack *f*, which it does; *CDE* should lack *f*, which it does; and *CdE* should lack *f*, which it does. (*CdE*, according to the theory, arises from either of two heterozygotes, *cDE/Cde* or *CDE/cde*, both involving a second order chromosome.) An occasional *cde* should lack *f*, and we have found one. A minority of *cDe* should lack *f*, but we have not found an example among 21 unrelated *cDe* chromosomes. An occasional *CDe* and *cDE* should have *f*, but these we have not yet encountered; a possible difficulty in their recognition will be mentioned below.

Other Possible Interpretations.—The possibility had to be considered that *f* is a new kind of blood-group antigen, a blended antigen, made up of *c* and *e* and produced only when their genes are together on *the same* chromosome. This was against all our experience. It was, for example, difficult to believe that *c* and *e* could have a fundamentally different effect when present in *Cde/cdE* and in *CdE/cde;* it was even more difficult to believe that Mr. Cl., who is *CDe/cDE*, could have made an antibody against a blend of two antigens both components of which he himself possessed. The possibility was ruled out when a chromosome *cde* lacking *f* was found (family 19, table 3).

Two other possibilities, much more difficult to exclude, are: (*a*) that *f* is an allele of *E* and *e*, say e^f, and that the common chromosomes are cde^f, cDe^f, *CDe*, *Cde*, *cDE*, *cdE*, etc.; and (*b*) that *f* is an allele of *C*, *c* and C^w, say c^f, and that the common chromosomes are c^fde, c^fDe, *cDE*, *cdE*, *CDe*, C^wDe, *Cde*, etc. They are difficult to exclude because there is evidence, to be given below, that most of our anti-*e* and some of our anti-*c* sera also contain anti-*f*.

We feel that the true explanation is unlikely to lie in either of these two possibilities for both require a number of very unusual qualifications of what we already know about *Rh* antisera. Further discussion over these two possibilities can be postponed since both should be excluded when the existence of chromosomes *cDE* and *CDe* "positive" with anti-*f* is proved, or when anti-*F* is found.

THE CONSEQUENCES OF ANTI-*f*

Phenotype Distinctions.—Anti-*f* does not promise to be of any great practical importance in *routine Rh* blood-group work. Certainly it splits

off *CDE/cde* and *CDE/cDe* (anti-*f* positive) from *CDe/cDE*, *CDe/cdE* and *Cde/cDE* (anti-*f* negative); it also separates *CdE/cde* (anti-*f* positive) from *Cde/cdE* (anti-*f* negative). These are hardly day-to-day problems though all these distinctions have been made in practice. Indeed the third apparent *Cde/cdE* tested with anti-*f* was positive and was thereby shown to be the very rare *CdE/cde*, and this was subsequently confirmed by the investigation of the family.[6]

A curious fact is that this one serum, anti-*f*, can distinguish between *DD* and *Dd* as competently as can the four sera anti-*C*, anti-*c*, anti-*E*, and anti-*e* combined. However it should be said that the use of the latter four antisera has a slight advantage in permitting a better estimate to be made of the error involved in the guess at the genotype; for example the phenotype *CDe/CDe* is less likely to be the genotype *CDe/Cde* than is the phenotype *cDE/cDE* to be *cDE/cdE*. Nevertheless, if anti-*f* becomes more generally available, this application will be of some importance in testing the blood of fathers of children with hemolytic disease of the newborn.

Failure to Make Anti-f in Volunteers.—Some time ago, in collaboration with Dr. B. H. Kirman, 34 *CDe/cDE* volunteers were given four intravenous injections, at intervals of three months, of 1 ml. of *cde/cde* blood. We hoped to make anti-*d* but in this we were disappointed; on looking back we should have been doubly disappointed, for our tests showed that anti-*f* was not made either.

The Need to Look for Anti-F.—If anti-F exists, as we presume it does, it will be difficult to identify with the test cells commonly available. It can be deduced from the left-hand column of table 2 that anti-*F* will give the reactions of a mixture of anti-*C* + anti-*E*. Anti-*F* could be made by *cde/cde* or by *cDe/cde* persons; if made by the former it would probably be mixed with anti-*D*. The most hopeful way to find anti-*F* is to test sera thought to contain anti-*C* + *E*, or anti-*D* + *C* + *E*, with cells of the genotype *cdeF/cdef*. So far we have tested five such sera but without success.

Anti-f in Anti-e and Anti-c Sera.—The sample of blood of the genotype *cdeF/cdef* was recognized early in the work on anti-*f*. The *e* and *c* antigens in this blood were thereupon titrated with anti-*e* and anti-*c* sera and the results showed that most of these sera behaved in saline as if they contained some anti-*f*, while in albumin their behavior was that expected of anti-*e* and anti-*c*. That is to say in saline the *cdeF/cdef* cells reacted much less strongly than did *cdef/cdef* cells, while in albumin the reactions were about equal. The effect was most marked with our anti-*e* sera: one of them, Br., absorbed 4 times with *CDe/cDE* cells left anti-*f* practically free from anti-*e*.

This evidence of two antibodies in the anti-*e* serum Br. explained a

puzzle of some years' standing. From 1946 to the end of 1949 the Blood Group Research Unit was doing comparative titrations with this serum. There was an interval of seven months, from October, 1948, to May, 1949, when no titrations were done. It was noticed, but not understood, that before this interval *CDe/cDE* cells scored more than *cDE/cde* cells but that after the interval *CDe/cDE* scored much less than *cDE/cde*. This change was reflected also in titrations with *cde/cde*, *CDe/cde*, and *CDe/CDe*. The scores are shown in table 5; they are strictly comparable wherever the ratio symbol is used. It is now clear that either the saline anti-*e* was deteriorating, perhaps because of storage, exposing an anti-*f* component or that the successive immunizations of Mr. Br. were stimulating anti-*f* while making the anti-*e* incomplete.

We were somewhat disturbed by the frequent presence of anti-*f* in these sera until we remembered that it was not until anti-*C* was discovered that so many anti-*D* sera were realized to be anti-*D* + *C*.

TABLE 5

CHANGE IN THE BEHAVIOR OF THE ANTI-*e* SERUM, BR.: SCORES FOR COMPARATIVE TITRATIONS IN SALINE

			cDE/cde		*CDe/cDE*	
Before October, 1948			742	:	864	
From May to December, 1949			205	:	43	
			Ff		*FF*	
	cde/cde		*CDe/cde*		*CDe/CDe*	
Before October, 1948	85	:	70	:	64	
From May to December, 1949	506	:	288	:	171	
	ff		*Ff*		*FF*	

It is probably because our anti-*e* and anti-*c* sera contain anti-*f* that we have not been able to identify *cDEf/cDEF* or *CDef/CDeF; cDEf/cDEF* would presumably be agglutinated by the anti-*f* in our anti-*e* sera and would therefore be called *cDE/cde*, and similarly *CDef/CDeF* would be labeled *CDe/cde* if the anti-*c* serum contained anti-*f*. This occasional wrong diagnosing of *cDE/cDE* and *CDe/CDe* would not show as discrepancies in family studies if the same anti-*e* and anti-*c* sera had been used on all members of the family.

It is clear that anti-*e* and anti-*c* sera are now exposed to investigation from a new angle.

The most important consequence of the recognition of *f* is that it must be taken into account in any comprehensive consideration of the *Rh* groups.

Summary.—The reactions of a "new" *Rh* antibody, anti-*f*, are described; they can most simply and conservatively be explained by the posulation of a fourth allelic series of antigens, *f* and *F*. The usual chromosome combinations, in white people, are: *cdef, cDef, cD^{u}ef, CDeF, cDEF, C^{w}DeF, CdeF, cdEF, CD^{u}eF, cD^{u}EF, CDEF, CD^{u}EF, C^{w}deF, CdEF, cDE^{u}F,*

C^wDeF, and C^wdEF. Certain less usual combinations are expected: of these *cdeF* has been found, *cDeF*, *CDef*, and *cDEf* are yet to be found.

Anti-*f* is commonly present in anti-*e* and anti-*c* sera.

Anti-*F* is presumed to exist but it will be somewhat difficult to identify.

Acknowledgments.—We are very grateful to the following people who have helped in the investigation of anti-*f* by sending us samples of blood, often of rare *Rh* genotypes: Dr. C. C. Bowley, Dr. C. Cameron, Mr. I. Dunsford, Dr. Jean Grant, Mr. C. J. Hasler, Dr. J. F. Hayward, Dr. C. A. Holman, Dr. Avril Johnstone, Dr. B. H. Kirman, Dr. P. L. Mollison, Dr. G. Owen, Dr. J. R. E. Richardson, Dr. G. H. Tovey, Dr. J. N. Walton, Dr. C. W. Wrigley, Dr. W. Weiner, and Dr. A. S. Wiener.

The grouping, other than *f*, of almost all the samples was done by Miss Joan Thompson, Miss June Cummings, and Miss Jean Noades of the Blood Group Research Unit; for most of the antisera they used we are once again indebted to Dr. A. E. Mourant of the Blood Group Reference Laboratory.

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*FURTHER STUDIES ON THE EFFECTS OF CARBON DIOXIDE AND OXYGEN ON THE FREQUENCY OF X-RAY INDUCED CHROMOSOME ABERRATIONS IN TRADESCANTIA**

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Previous studies have shown that exposure to carbon dioxide during irradiation markedly increases the radiosensitivity of cells.[1–5] It has also been demonstrated that the concentration of oxygen present during irradiation has a profound effect on the radiosensitivity (e. g., Giles and Riley[6]). The experiments to be reported examine the interaction between these two gases and further define the mode of action of carbon dioxide in increasing radiosensitivity.

Material and Methods.—*Tradescantia paludosa* Anderson and Woodson was used in all experiments. Cytological observations of chromosomal

aberration types were made 5 days after irradiation. The principal types of aberrations analyzed at this period are double interchanges (dicentrics and centric rings).

The experimental methods were essentially the same as those described previously.[5] The gases employed were obtained in commercial cylinders and assayed as follows: Nitrogen (Airco), 99.5% N_2 plus 0.5% O_2; Carbon Dioxide (Liquid Carbonic Co.), 99.8% CO_2, 0.05% N_2, 0.009% O_2, 0.07% H_2O. The inflorescences were placed inside $3^1/_2$-liter lucite chambers initially filled with air. In one series of experiments the chambers were first evacuated and then refilled with specific gases at specific pressures. In a second series, the chambers were not evacuated; specific positive pressures of the gas under consideration were superimposed on the atmosphere of air initially present in the chamber.

TABLE 1

FREQUENCIES OF DICENTRICS AND CENTRIC RINGS INDUCED IN TRADESCANTIA MICROSPORES EVACUATED AND THEN X-RAYED IN VACUO OR IN MIXTURES OF AIR AND CARBON DIOXIDE. ALL DOSAGES ADMINISTERED AT A CONSTANT INTENSITY OF 40 R/MIN. FOR 10 MINUTES

EXPERIMENTAL			CONTROL		
GAS MIXTURE (ATMOSPHERES) CO_2	AIR	DICENTRICS AND RINGS, %	GAS MIXTURE (ATMOSPHERES), AIR	DICENTRICS AND RINGS, %	EXPERIMENTAL AS PER CENT OF CONTROL
0	0	0.9	1	6.4	14
0.33	0	2.0	0	1.7	117[a]
0.33	0	1.9	0	1.8	106[a]
0.33[b]	1	13.4	1	7.3	184
1	0	1.0	0	1.3	77[a]
1[b]	1	23.8	1	7.7	309

[a] Differences of this order of magnitude between experimental and control with aberration frequencies shown here are not statistically significant for the number of chromosomes counted (i.e., 3000).

[b] These preparations were not subjected to preliminary evacuation.

After the desired gas mixture had been established within the chamber, the latter was sealed and was maintained at 25°C. in the dark for a specified period. It was then placed in the x-ray machine alongside an identical chamber containing the dosimeter. Irradiation was performed at room temperature in subdued light. This procedure in each experiment was carried out twice—once with the experimental gas (usually CO_2) and once with the appropriate control gas (usually air). Within 30 minutes after irradiation the chambers were opened and the influorescences returned to air.

Ten to 15 inflorescences were exposed in each chamber. Five days following irradiation as many slides as possible were prepared. Three thousand chromosomes were counted in each control and experimental group. The scoring was performed by one investigator (E. D. K.).

Results.—In an effort to examine the interaction between carbon dioxide and oxygen in conditioning radiosensitivity, the following experiments were carried out. The chambers containing the influorescences were evacuated for $3^1/_2$ minutes and then either maintained in the evacuated state or refilled to a desired pressure with a particular gas. Fifteen minutes after the initial evacuation the chambers containing inflorescences were irradiated for 10 minutes at a constant rate of 40 r/min., after which the chambers were slowly decompressed and the inflorescences returned to air. The results summarized in table 1 permit the following conclusions:

1. Exposure to a vacuum during irradiation markedly decreases the aberration frequency below that encountered in control preparations irradiated in air. This result is in agreement with numerous observations by other workers.[6]

2. Exposure to $^1/_3$ of an atmosphere of carbon dioxide plus 1 atmosphere of air greatly increases the aberration frequency above that of controls irradiated in air.

TABLE 2

FREQUENCIES OF DICENTRICS AND CENTRIC RINGS INDUCED IN TRADESCANTIA MICROSPORES EXPOSED TO X-RAYS IN AIR AND THEN COMPRESSED WITHIN 18 SECONDS WITH CARBON DIOXIDE. ALL DOSAGES ADMINISTERED AT A CONSTANT INTENSITY OF 200 R/MIN. FOR 2 MINUTES

	GAS MIXTURE (ATMOSPHERES)		DICENTRICS AND RINGS, %	EXPERIMENTAL AS PER CENT OF CONTROL
	CO_2	AIR		
Experimental.....	0.33	1	7.0	96[a]
Control.....	..	1	7.3	..

[a] Differences of this order of magnitude between experimental and control with aberration frequencies shown here are not statistically significant for the number of chromosomes counted (i.e., 3000).

3. Irradiation in the presence of as much as 1 atmosphere of carbon dioxide in the absence of air is equivalent to irradiation in a vacuum.

Thus it appears that carbon dioxide acts synergistically with oxygen in increasing radiosensititivity. But the effect of carbon dioxide on radiosensitivity disappears when oxygen is absent. In short, it appears that carbon dioxide acts by modifying radiochemical reactions that involve oxygen; it affects the "indirect action" of x-rays and not the "direct action" (cf. Sparrow and Rubin[7]).

In a second series of experiments we sought to determine whether carbon dioxide acts at the instant of irradiation or subsequent to radiation. To this end, the inflorescences were first irradiated at 200 r/min. for 2 minutes. Then, within 18 seconds after irradiation, they were compressed with $^1/_3$ of an atmosphere of carbon dioxide. After 60 minutes of exposure to the carbon dioxide, the chamber was decompressed and the inflorescences re-

turned to air. A control group was treated in identical fashion save for omission of the compression with carbon dioxide. Table 2 shows that post-irradiation treatment with carbon dioxide had no detectable effect. Consequently, we conclude that carbon dioxide exerts its action at the time of irradiation. Moreover, since carbon dioxide and oxygen act synergistically, it follows that oxygen acts at the time of irradiation.

Figure 1 records the effects of mixtures of one atmosphere of air plus varying pressures of carbon dioxide. The results of the present study are plotted along with those previously reported.[5] In all these experiments the air-filled tanks and the inflorescences therein were compressed with carbon

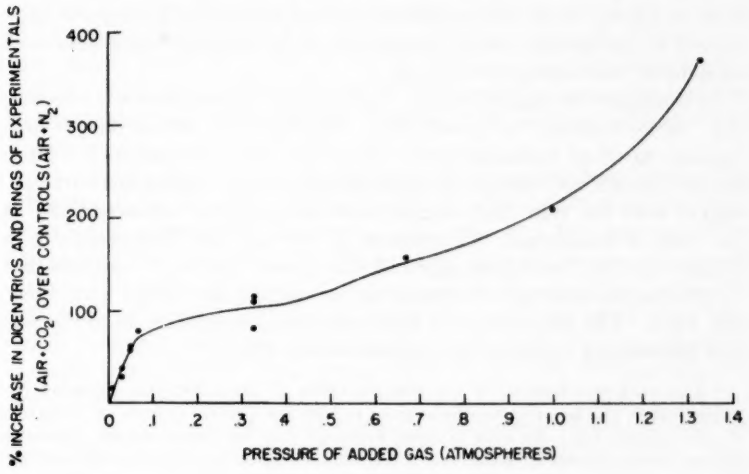

FIGURE 1

Curve showing the effect of mixtures of one atmosphere of air plus varying pressures of carbon dioxide or nitrogen on the incidence of x-ray-induced dicentrics and centric rings in Tradescantia microspores.

dioxide (or nitrogen in the case of controls) for 1 hour prior to irradiation. The inflorescences were then irradiated for 10 minutes at a constant rate of 40 r/min. Within 30 minutes after irradiation the inflorescences were returned to air. As previously noted[5] the results show that the greater the carbon dioxide concentration (up to at least $1^1/_3$ atmospheres of carbon dioxide), the greater the increase in aberration frequency. Moreover, carbon dioxide concentrations of less than 1 per cent of an atmosphere appear to cause a significant increase in aberration frequency.

The relation of carbon dioxide pressure to radiosensitivity in Tradescantia (Fig. 1) differs strikingly from that observed in fern spores and

Drosophila eggs by Zirkle.[2-4] A complex "wave-form" relation between carbon dioxide concentration and radiosensitivity was reported by this investigator: certain concentrations of CO_2 increased radiosensitivity and other concentrations decreased radiosensitivity. Thus, in the case of Drosophila eggs, carbon dioxide tensions between 16 and 21% and above 64% of an atmosphere exerted a protective effect against radiation damage.[3]

Our experiments with Tradescantia reveal no such "wave-form" relation between carbon dioxide concentration and radiosensitivity. To the contrary, radiosensitivity underwent a steady, albeit non-linear increase, as the carbon dioxide concentration increased. Thus carbon dioxide affects chromosome aberrations in Tradescantia in a manner strikingly different from its effects on the radiosensitivity of fern spores and Drosophila eggs. Indeed it appears that carbon dioxide may have widely different influences on different radiobiological systems.

In conclusion we suggest that in Tradescantia carbon dioxide is affecting the "indirect action" of x-radiation. Furthermore, carbon dioxide here appears to affect radiosensitivity only at the time of irradiation. Since carbon dioxide and oxygen act synergistically these observations are consistent with the view that oxygen exerts its action on radiosensitivity at the time of irradiation. Comparison of our data for Tradescantia with Zirkle's data for Drosophila eggs and fern spores leads us to conclude that in different radiobiological systems carbon dioxide may act in very different ways. The information at hand permits no conclusion as to the precise biochemical nature of the carbon dioxide effects.

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GENE ACTION IN RELATION TO GROWTH AND DEVELOPMENT. I. PHENOTYPIC VARIABILITY

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Among the most fundamental problems in biology belongs the action of the gene. The gene, however, is elusive since no direct approach is possible: the gene can only be recognized by its effect. These effects may be used either in their final expression—the heritable characters—and then we are in the domain of classical genetics. Or we can try to infer from the final and intermediate steps of gene action what lies at the beginning of this chain. This is the approach of both the biochemist, the embryologist, and the physiologist. The successes of the biochemist in approaching the action of the gene are spectacular, such as in the analysis of biosynthesis in Neurospora.

The physiologist or embryologist cannot hope to contribute much in the direction of the chemistry of gene action, since they are dealing with processes rather than specific biochemical reactions. But they can contribute in several other directions. On the one hand they are dealing with growth and development, which cannot be expressed in chemical terms as yet but which are of such importance in inheritance. On the other hand they stress the quantitative side of biological processes more than the biochemist, who, in his analysis of the action of the gene, largely stresses the pathways of gene action.

The following discussion will deal primarily with physiological observations which have a bearing on the quantitative side of gene action. This problem has many different aspects. The problem of practically absolute uniformity in gene reproduction will not be dealt with.

In gene action, we find absolute reproducibility in ontogeny, in which the sequence of events and of organs meets only very seldom with a slight deviation in timing, but the number and order of, for example, flower parts in the pea flower are practically without variability.

Not only the qualitative aspects, but also the quantitative side of growth is genetically controlled. Here we meet with a much greater variability: Even within a pure line or clone there is considerable variation in size or weight or any other quantitative character.[1] Two factors contributing to this phenotypic variability have been recognized. In the first place, the environment has a great effect on the expression of quantitative characters. Hormonal control of growth insures proportionality between the different parts of the organism: when for some reason one part is small, most other organs are proportionately reduced in size. The absolute di-

mensions of the organism are therefore less directly gene-controlled. The proportions, however, are under gene control. Yet the size of an organism under a specific set of growing conditions, and particularly its maximal size, is definitely an inheritable character.

In the second place, all cellular reactions occur on a microscopic scale, where the molecular structure of matter starts to interfere with the regularity of classical statistical physics. This has been pointed out by many physicists,[2-4] and was very clearly expressed by P. Jordan in 1936: "Wide applicability of macrophysical laws to organisms is unquestioned; yet there are good grounds to believe that the basic life processes do not belong in the realm of macrophysics any more. The macroscopical processes occurring in the larger parts of the body are governed by considerably more delicate processes. The ultimately controlling reactions are generally of atomic dimension." This leads Jordan to his "amplifier" theory of organisms, in which he stresses the fact that in organisms the basic controlling "reactions of atomic dimensions" such as gene reactions, are amplified to macroscopic observability by cellular processes. "Genetics provides the most convincing and widest basis for the thesis, that organisms are not macrophysical, but microphysical systems."

Continuing his argument, Jordan then states that unstability, or inconstancy, is a fundamental characteristic of quantum physics and that actually inconstancy occurs in biology as well, as the statistical nature of the Mendelian laws shows. From this, one might conclude that when genes control development, one would expect a fundamental variability in organisms. This thesis was actually elaborated by Otto Rahn[5] who calculated from the observed variability in biological material (in his case bacteria) the numbers of genes involved. He obtained figures ranging from 100 to 1000, which agree with estimates made of the number of genes occurring in organisms using other premises.

Probably few other biologists rationalized their acceptance of phenotypic variability as due to control of growth and development by atomistic reactions in the sense of Jordan (see, e.g., the criticism of Bünning).[6] Yet we find that not only geneticists, soil scientists and agriculturists, who have to work with variable material of which they cannot reduce the variability, but also biologists, have adopted statistical analysis as the general method of coping with variability in biological material. This indicates to what extent phenotypic variability was accepted as a basic property of living matter by biologists as a whole. If phenotypic variability had been considered as being largely due to environment, more serious efforts would have been made to control the external environment of growing plants.

Experience gained in the Earhart Plant Research Laboratory shows that environment rather than the atomistic nature of biological reactions is responsible for phenotypic variability. It was generally observed that plant

material grown under the controlled conditions of this laboratory was less variable than similar material grown in an ordinary greenhouse. This was of practical importance in the design of experiments, in which fewer objects could be used to obtain the same significance, even to the extent that only four plants are now used per treatment in many experiments. Justification for this reduction in size of sample in experiments is found in figure 1. This shows the effects of an increase in uniformity of growing

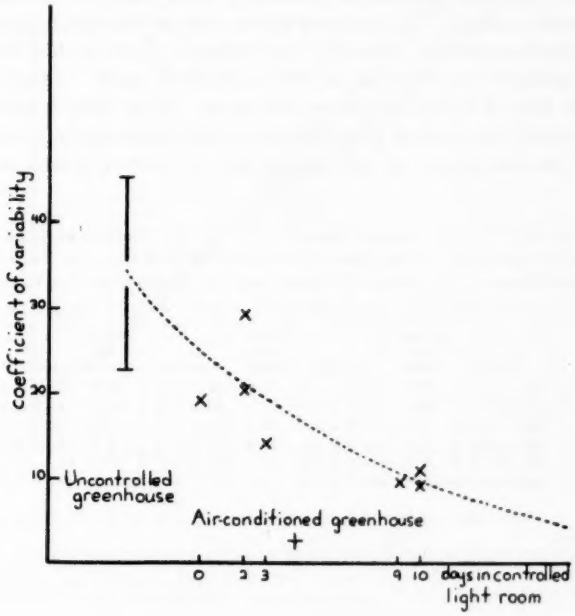

FIGURE 1

Coefficient of variability of dry weight of tomato plants grown in either an ordinary greenhouse (*left*) or in an air-conditioned greenhouse (*right*). The latter plants were kept for 2, 3, 9, or 10 days (abscissa), prior to weighing, in controlled light rooms with even more uniform environment.

conditions on variability in weight of young tomato plants, when they are grown with different degrees of environmental control. In most plant material we find that the coefficient of variability—when the standard deviation of the mean is expressed in per cent of the average—amounts to anywhere between 20 and 40 per cent. In the Earhart greenhouses this variability is reduced, usually to about 15 per cent. Yet, when similar plants are grown in temperature- *and* light-controlled rooms, their coef-

ficient of variability is decreased to about 8 per cent. The details of these determinations will be published later.

At present, the question cannot be answered how much of the remaining 8 per cent variability is innate, and how much is imposed by inconstancy of growing conditions. In the following observations the coefficient of variability in pea plants decreases to below 4 per cent (table 1) and it is very likely that further work will reduce this still more.

We can view this problem of variability from many other angles. If growth were controlled by processes whose rates are subject to appreciable random fluctuations, the variability of a batch of plants should remain the same or perhaps even increase as they continue to grow. This is not actually the case as field observations will show: Even though germination may be variable in a wheat or corn field and the seedling stand may consist of fairly uneven plants, a well-tended field of mature wheat or corn is

TABLE 1

LENGTH IN MM. OF PEA PLANTS, GROWN IN A 16-HR. PHOTOPERIOD AT 17°C. IN DIFFERENT INTENSITIES. EACH GROUP CONSISTED OF 9 PLANTS; IN EACH CASE THE STANDARD ERROR OF THE MEAN IS GIVEN AND THE COEFFICIENT OF VARIABILITY

	VINCO, 1500 FT.-C.		VINCO, 1000 FT.-C.		VINCO, 500 FT.-C.		KRONBERG, 1500 FT.-C.	
DATE	LENGTH	COEFF. OF VARIA.	LENGTH	COEFF. OF VARIA.	LENGTH	COEFF. OF VARIA.	LENGTH	COEFF. OF VARIA.
23 VII	34.3 ± 1.4	11.5	35.8 ± 1.3	10.3	37.5 ± 1.5	11.3	32.4 ± 2.0	17.4
25 VII	51.8 ± 2.0	10.9	49.3 ± 2.0	11.5	53.3 ± 2.3	12.2	45.9 ± 2.7	16.5
31 VII	107.2 ± 2.6	6.8	107.8 ± 2.2	5.9	104.2 ± 2.7	7.3	80.9 ± 5.2	18.1
7 VIII	189.4 ± 3.0	4.5	191.3 ± 3.1	4.8	163.5 ± 4.1	7.1	142.4 ± 7.7	15.3
12 VIII	235.0 ± 3.0	3.6	248.9 ± 4.0	4.6	205.0 ± 3.9	5.4	.....	..
13 VIII	249.6 ± 3.0	3.4	268.1 ± 4.0	4.2	223.5 ± 4.2	5.3	194.9 ± 6.3	9.1
Dry wt. in mg.	870 ± 23	5.2	857 ± 37	12.1	415 ± 9	6.1	762 ± 53	20.0
Actual increase in length from 31 VII to 13 VIII	142.3 ± 2.6	..	.....	..	.....	..	114.0 ± 3.6	..

surprisingly uniform. The following set of measurements substantiates this field observation. Groups of "Vinco" and "Kronberg" peas were grown in three different light intensities: 500, 1000, and 1500 ft.-c., and as they grew, the distance to the artificial lights was adjusted so that their tops continued to receive the same intensities.

To start with, groups of nine plants were selected to be as uniform as possible in respect to size and form. They were measured from time to time, and table 1 and figure 2 show the results. The variability was small to start with in all three groups; in absolute figures the variability increased least in the peas growing in the highest light intensity. But the relative variability decreased very much—to less than one-half of what it originally was. Because the variability in these peas was so small to start with, the causes for its decrease are not so clear. In the

Kronberg variety which was more variable at the start, variability remains much greater, but not because the longest plants continue to grow fastest.

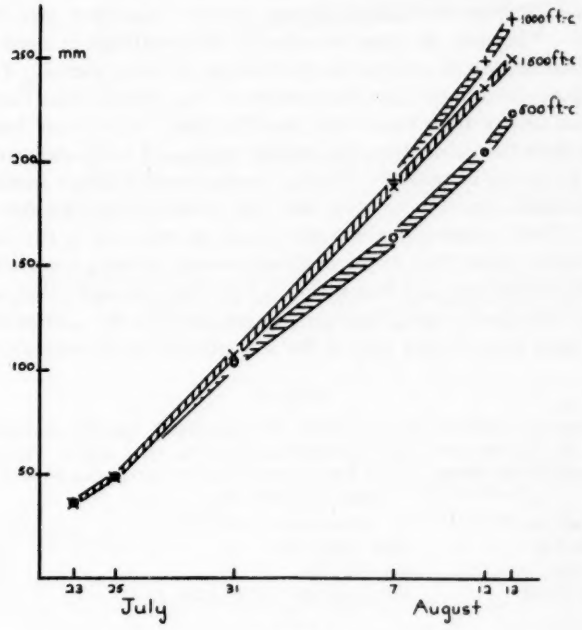

FIGURE 2

Length of Vinco pea plants grown in a 16-hour photoperiod at 17°C. in different light intensities. Variability of each group of plants is indicated by a 2 × standard error.

For it is seen that the variability of the length increase during the last fourteen days is less than the previously existing variability (the largest

TABLE 2

GROWTH OF PEA PLANTS DEVELOPING FROM SEEDS OF DIFFERENT WEIGHT. AVERAGE OF 25–35 PLANTS PER GROUP

WEIGHT OF DRY SEED, MG.		GROWTH IN MM.			TOTAL LENGTH,
AVERAGE	RANGE	AUG. 13–18	AUG. 18–21	AUG. 21–26	AUG. 26
180	130–199	24.2	36.5	73.5	134.2
230	201–246	22.2	37.5	77.7	137.5
330	298–377	20.7	31.2	84.0	136.0

differences on July 31 were 54 mm.; on August 13, 69 mm., whereas for the length increments they were only 34 mm.).

These measurements show that the growth rate of these peas does not depend on the preceding conditions, and that differences within a well-selected strain are neither genetically nor phenotypically conditioned. Differences between individuals do not become magnified, but decrease relatively. Therefore we have to consider the possibility of a self-regulatory mechanism which adjusts the growth rate of these plants. This also follows from the observation that originally the plants from bigger pea seeds grow slower than those from smaller ones; after some time they catch up with the plants from the smaller seeds and reach the same overall size, as shown in table 2. Similar results were obtained when plants were arranged according to their size and growth rate after one week's growth. Those growing fastest reduce their growth rate in the following eleven days to below that of the originally slower growing peas to such an extent that their final size was within 0.7 per cent of each other, whereas originally this group was 17 per cent larger (see Fig. 3). Even the very smallest peas (only 45 per cent of the largest) caught up with the others.

TABLE 3

COEFFICIENT OF VARIABILITY OF TOMATO PLANTS WHEN GROWN IN SUBOPTIMAL, OPTIMAL AND SATURATING LIGHT INTENSITIES AT 17°C. IN A 16-HR. PHOTOPERIOD. THE VARIABILITY IS BASED ON THE DRY WEIGHTS OF 20 CONTAINERS WITH 20 PLANTS EACH, AS IN FIGURE 1

LIGHT INTENSITIES, FT.-C.	COEFFICIENT OF VARIABILITY	AVERAGE, %
500–700	12.0; 12.5; 14.2	12.9
900	8.4; 8.9; 8.9	8.7
1100–1500	9.8; 10.2; 10.7; 11.6; 12.0; 13.3	11.3

This was especially evident when a correction was made for the physiological age of the small plants—which is about two days less than that of the other peas—then their growth rate fell within the range of the originally taller plants.

In connection with variability of experimental material another observation has to be recorded. Under optimal growing conditions, when rate of elongation is greatest, the coefficient of variability is least (see Fig. 4). This was found in young pea plants grown at different temperatures. It is interesting to note that in the youngest stage, the growth rate is unaffected by temperature over the 15.5° to 26°C. range. Only below 15° is it controlled by a process with a high temperature coefficient. And it is just where growth is presumably limited by a chemical process that variability increases.

In tomato experiments it was also found that under most nearly optimal growing conditions the variability of the plants was the least; therefore, also at the greatest rates of growth. For instance, the average dry weights of tomato plants grown like those in figure 1 showed a coefficient of varia-

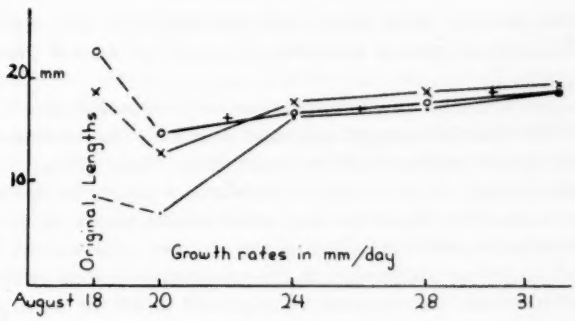

FIGURE 3

Growth rates of Vinco peas in mm./day (ordinate). Plants were divided into 3 groups: the shortest, tallest, and intermediate, and the growth of each group was followed for 2 weeks.

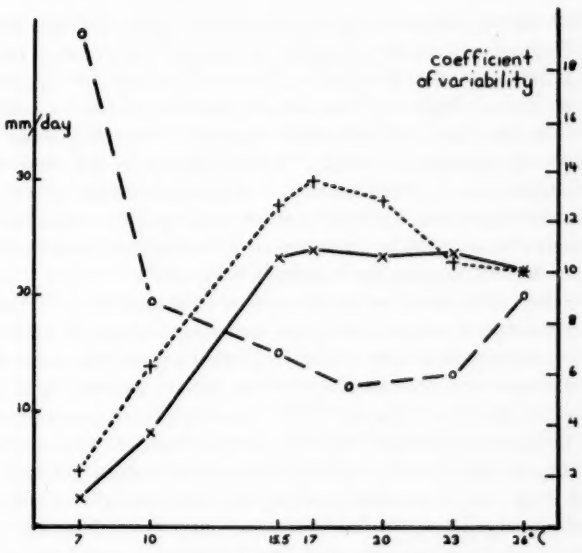

FIGURE 4

Growth rates (left ordinate, mm./day) of Vinco peas at different temperatures (abscissa) for the period of 5–13 days after planting (crosses and solid line) and 13–20 days after planting (plusses and dotted line). Coefficient of variability (right ordinate) of the growth rate from 5 to 13 days shown in circles with broken line.

bility of 12.9 per cent when grown at suboptimal light intensity, and of 11.3 per cent at supraoptimal intensities, but of 8.7 per cent at the optimal intensity (table 3).

We can draw a number of conclusions from these observations:

(1) Variability in the material described is smaller than ordinarily supposed to be typical for genetically uniform plants. The selected examples are not exceptional, however, and variability of plants in the Earhart Laboratory is generally much less than when similar plants are grown under uncontrolled conditions. This is due to two independent effects: (*a*) through proper air distribution in the greenhouses and controlled light rooms, the individual plants are more nearly all under the same growing conditions, which they are not, e.g., on an ordinary greenhouse bench where the plants in the center have much less air circulation and therefore are subjected to higher temperatures during the day, and (*b*) under optimal growing conditions variability is also smaller (Fig. 4); with the great variety of growing conditions in the Earhart Laboratory plants in general can be grown under more nearly optimal conditions and consequently will be less variable.

(2) From figure 1 it can be seen that in genetically uniform material, the upper limit of any innate variability is around 5 per cent. The additional variability usually observed is imposed largely by uncontrolled growing conditions. There are two explanations for this low variability.

(3) On the one hand, it is possible that low variability is an innate characteristic of biological material. This seems to be the view of both Bohr and Schrödinger. Bohr[2] states: "The explanation of the properties of living organisms certainly lies in their specific organization, in which typical atomic traits are interwoven with mechanistical traits to an extent which has no counterpart in inorganic nature."

(4) On the other hand, we could assume that growth is variable like any physicochemical process, but that superimposed on it is an autoregulatory process, which corrects any deviation imposed on it by chance. This would seem to be substantiated by the data of figures 2 and 3, from which it follows that the originally slower growing plants grow faster afterward, but in later development this was less pronounced: It took the peas (shown in Fig. 3) which were growing slowest from August 22 to 26 three days to catch up with the fastest growing ones and even then they did not surpass them (originally 23.8 and 27.4 mm./day; next three-day period, 24.0 and 28.7 mm./day; last four-and-a-half days, 28.7 and 28.7 mm./day). Therefore, although there is a tendency for growth of the slower growing plants to pick up, there is no *immediate* response in all cases, and the self-regulatory mechanism is not very intimately tied up with the growth process itself.

(5) Is it possible to imagine a process which controls growth which

could explain the phenomena described in this paper? From figure 4 we can conclude that at the optimal growth rate a diffusion process, largely independent of temperature, controls growth in young peas. Along a very different line of reasoning,[7] it was concluded that diffusion within a cell between the nucleus and cytoplasm is the limiting process in growth.

(6) Such a diffusion process is slower when the cells or organs are larger. Thus when faster growth has resulted in larger cells in the growing point, this would automatically reduce the growth rate. This is just the opposite of control by chemicals, where a larger original structure would tend to increase the supply of hormones or other growth factors and would result in faster growth. We would expect, then, the larger individuals to be both the result of and to result in a greater growth factor supply. Therefore through chemical control of the growth rate, original small differences in size would tend to become magnified, whereas just the opposite occurs (Figs. 2 and 3 and table 2). By the simple assumption that growth is controlled by an intra- or intercellular diffusion process, we can explain a decrease in growth rate of the growing points which have become larger than the average and vice versa.

(7) How can we tie the observed phenomena in with genes? The gene theory was based upon the discontinuity of the hereditary process. The distribution of characters over the offspring is a typical quantized phenomenon, explained by the presence in cells of a few discrete units or genes. The same genic concept can account for effects of ionizing radiation on organisms (see, e.g., Timofeeff-Ressovsky and Zimmer[8]); through these radiation effects the approximate size of the gene can even be calculated.

There is, of course, no doubt that growth and the growth rate in plants is dependent upon the hereditary mechanism. For each pea variety, although very uniform within itself, differs from most other varieties in its growth rate. Yet, since the growth rate is so remarkably uniform from plant to plant, we must conclude that either the growth rate is only very indirectly dependent upon genes, or that growth is controlled by hereditary units which are present, not in twofold, but in very large numbers within each cell.

The former possibility resolves itself into two mechanisms: Either the laws of quantum mechanics do not hold for the reproduction and physiological effects of genes, as Schrödinger[4] suggests, or the gene becomes multiplied in each cell before it exerts its effect. In practice, the latter mechanism becomes almost identical with the assumption of polygenes controlling quantitative processes inside the cell. But whereas formerly the nature of polygenes could only be inferred from the distribution of the quantitative characters over the offspring, it should now become possible to study this problem by the quantitative effect of the hereditary mechanism upon the growth process within an individual. In subsequent papers this will be analyzed in more detail.

The physiologist and embryologist are now in the uncomfortable position where they will have to explain why genes, which were just proved by the geneticist and biophysicist to be following quantum mechanics, do not impart the degree of variability upon growth and development which one would expect from quantum mechanics. We can view this difficulty also from another standpoint, however. If the uncertainty principle of quantum mechanics did hold for gene reproduction and gene effect, organic life in its present form would be impossible, because there would be only statistical reproducibility of organisms, and very soon life would have reverted to the more stable and probable inorganic state. Only because development does not follow the laws of quantum mechanics can the less probable condition of life be continued indefinitely. The reduced variability of genetically uniform plant material when grown under controlled conditions is therefore nothing exceptional; it is the logical consequence of the rigid laws of organic development which fall outside the laws of statistical or quantum mechanics. This has been expressed before by Schrödinger: "The unfolding of events in the life cycle of an organism exhibits an admirable regularity and orderliness unrivaled by anything we meet in inanimate matter."

Summary.—The major part of the commonly encountered variability of genetically uniform material (phenotypic variability) is not due to statistical fluctuations of the numbers of molecules on which development depends, but is largely caused by inconstancy of and irregularities in the external environment. The genetic homogeneity of pure lines can be translated under completely controlled conditions into phenotypic uniformity of plant material which exceeds anything observed thus far. The more optimal the growing conditions are, the smaller is phenotypic variability. When the standard deviation is expressed as per cent of the mean, variability in peas and tomatoes is regularly reduced to 2 per cent, and in the most favorable material it was as little as 1.2 per cent. Due to an autoregulatory mechanism phenotypic variability decreases in the course of development. All these facts lead to the conclusion that the laws of organic development fall outside the laws of statistical or quantum mechanics.

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[2] Bohr, N., "Licht und Leben," *Naturwiss.*, **21**, 245–250 (1933).

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[6] Bünning, E., "Physikalisch-chemische Grundlagen der biologischen Vorgänge," *Fortschr. Bot.*, **8**, 154–165 (1939).

[7] Went, F. W., "Physical Factors Affecting Growth in Plants" (in press), 1953.

[8] Timofeeff-Ressovsky, N. W., and Zimmer, K. G., *Biophysik I. Das Trefferprinzip in der Biologie*, Hirzel, Leipzig, 1947.

TRAVEL TIMES OF LONGITUDINAL WAVES FROM SURFACE FOCI

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Tables and graphs for travel times of seismic waves propagated through the earth are based on earthquake records. This requires accurate knowledge of the longitude and latitude of the corresponding epicenters, focal depths, and origin times. It is highly desirable to compare the resulting travel time curves and tables with results derived from experiments in which all four fundamental quantities are accurately known from independent data.[1] Seismologists are indebted to scientists and organizations for measurements of records and other corresponding information from which accurate travel times of longitudinal waves originating at known surface sources in the Pacific area could be found.

Residuals for 33 observed direct longitudinal waves, P, were calculated relative to travel times of Jeffreys and Bullen[2] for average continental surface foci, as well as relative to similar tables by Gutenberg and Richter.[3] The following average residuals (with standard errors) from the Jeffreys-Bullen tables[2] are found in four ranges of distances containing most of the observations:

Distances, deg.	35–45	50–65	68–79	80–91
Residuals, sec.	-1.7 ± 0.6	-3.9 ± 0.4	-1.3 ± 0.6	-1.1 ± 0.7

It has been suspected previously[1] that travel times from surface sources in the Pacific basin are about 2 seconds shorter than the corresponding times from continental surface foci due to differences in velocity in the surface

TABLE 1

CORRECTIONS TO THE TRAVEL TIMES OF JEFFREYS-BULLEN[2] (JB) AND GUTENBERG-RICHTER[3] (GR) FOUND FROM PACIFIC SURFACE SOURCES (S, REDUCED TO CONTINENTAL STRUCTURE) AND THE EARTHQUAKE OF JULY 21, 1952 (E); FOR DETAILS, SEE TEXT. Δ = ANGULAR DISTANCE

	Δ	30	40	50	60	70	80	90	100 deg.
JB	E	+1	+1	0	−1	0	+1	+1	+1 sec.
	S	+1	0	−1	−2	0	+1	+ ½	0 sec.
GR	E	−1	−2	−3	−3	−3	−1	−1½	−1 sec.
	S	0	−2½	−3	−4	−2	−1	−2	−2 sec.

layers. Consequently, 2 seconds were added to all residuals from Pacific surfaces. Averages based on graphs of the individual residuals similar to figure 1 are given in the lines marked S in table 1.

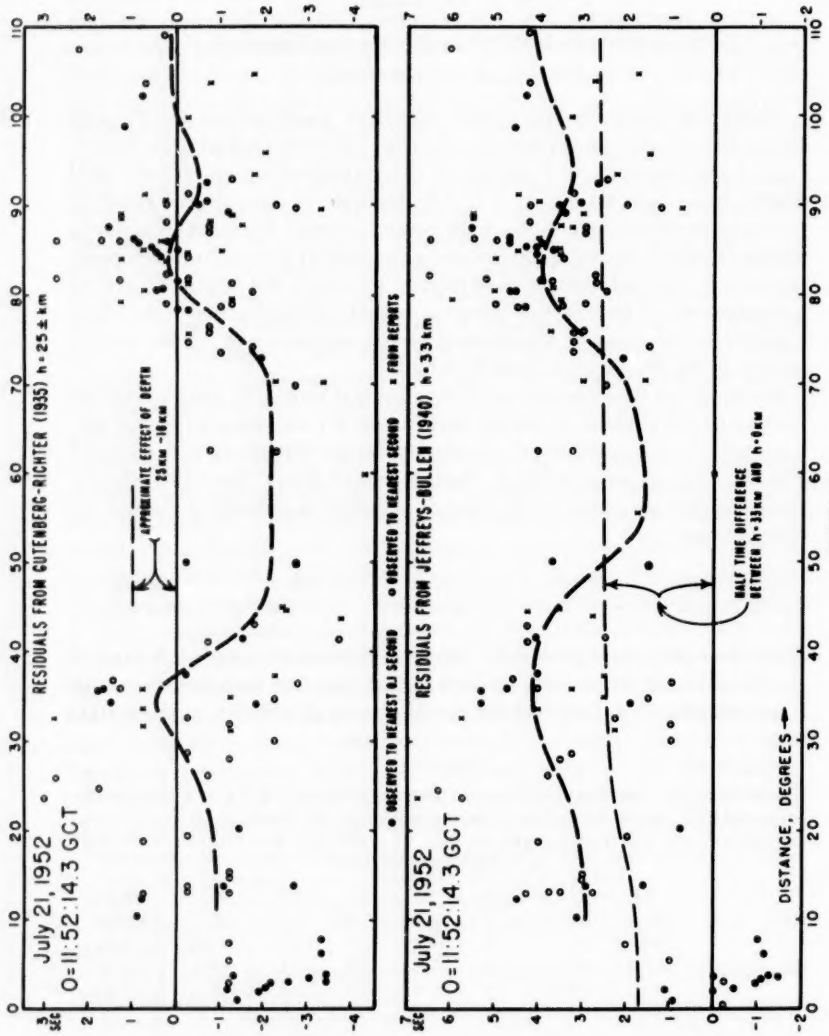

FIGURE 1

Residuals of arrival times of direct longitudinal waves, *P*, July 21, 1952.

Records of the Kern County, California, earthquake of July 21, 1952, gave an opportunity to calculate travel times from a well-located continental source. Over 150 stations all over the world responded to a request to mail originals or copies of their records to Pasadena. Readings of arrival times from 40 additional stations are available at the time of writing. Careful determination of the elements of this shock (to be described elsewhere) resulted in an origin time of $11^h52^m14.^s3$ with a standard error of about 0.2 second and an epicenter at 35°00′ North Longitude, 119°02′ West Latitude, probably correct within 3 km.

For all measured and reported arrival times of the direct longitudinal wave *P* travel times were calculated as well as the corresponding geocentric distances. Residuals from the travel times of Jeffreys-Bullen[2] for their focal depth "$h = 0.00$" (supposed to correspond to 33 km.) and from the travel times of Gutenberg-Richter[4] for a focal depth h of 25 ± km. were calculated for each reading and plotted as a function of distance in the respective two sections. Half the travel time differences between the curves of Jeffreys-Bullen for "$h = 0.00$" and "surface focus" are also indicated in the lower part of figure 1. Since the earthquake of July 21, 1952, probably had a focal depth of about 16 km., the deviation of the observed times from this curve corresponds approximately to the corrections to be applied to the Jeffreys-Bullen tables. Similarly, the differences in travel time between $h = 25$ and $h = 16$ km. is indicated in the upper part of the figure. It has been found previously[5] that the zero point of the Gutenberg-Richter travel times is about 1 second too early. To adjust for this difference 1 second would have to be added to all residuals in the lines *GR* of table 1.

Table 1 shows that the assumptions made, especially those for the difference between Pacific and continental velocities in the earth's crust, are good approximations.

From table 1 and the corresponding graphs (including figure 1) corrections to the *JB* and the *GR* curves were estimated for every degree and applied to the corresponding tables for surface focus. Table 2 contains a smoothed revised travel time curve for continental surface focus on the basis of these results.

The data for the Pacific surface sources contained five instances of *P″* (longitudinal waves through the outer and inner core) observed at distances between 110 and 140 degrees. Three of them showed two phases between 5 and 15 seconds apart. The earlier phases probably correspond to early *P″* waves observed in earthquake records; "these early readings are probably not due to longitudinal waves approximately following the laws of geometrical optics; a phenomenon of diffraction is probably involved."[6] For the five later impulses the residuals based on the tables of Gutenberg and Richter[7] for surface focus are very small if the hypothetical effect of Pacific structure is considered.

TABLE 2

TRAVEL TIMES OF LONGITUDINAL WAVES FROM CONTINENTAL SURFACE FOCUS; Δ = DISTANCE IN DEGREES

Δ	0	1	2	3	4	5	6	7	8	9
0	0:00	0:17.9	0:35.2	0:48.8	1:02.4	1:16.0	1:29.6	1:44.7	1:59.8	2:14.9
			13.6	13.6	13.6	13.6	15.1	15.1	15.1	15.1
10	2:30.0	2:45.2	2:58.0	3:10.8	3:23.6	3:36.4	3:49.2	4:02.0	4:14.5	4:26.5
	15.2	12.8	12.8	12.8	12.8	12.8	12.8	12.5	12.0	11.5
20	4:38.0	4:48.4	4:58.5	5:08.4	5:18.1	5:27.7	5:37.1	5:46.4	5:55.5	6:04.5
	10.4	10.1	9.9	9.7	9.6	9.4	9.3	9.1	9.0	8.9
30	6:13.4	6:22.3	6:31.1	6:39.8	6:48.5	6:57.1	7:05.6	7:14.0	7:22.4	7:30.8
	8.9	8.8	8.7	8.7	8.6	8.5	8.4	8.4	8.4	8.3
40	7:39.1	7:47.3	7:55.5	8:03.7	8:11.8	8:19.8	8:27.6	8:35.3	8:42.9	8:50.5
	8.2	8.2	8.2	8.1	8.0	7.8	7.7	7.6	7.6	7.5
50	8:58.0	9:05.4	9:12.7	9:19.9	9:27.0	9:34.1	9:41.2	9:48.3	9:55.4	10:02.5
	7.4	7.3	7.2	7.1	7.1	7.1	7.1	7.1	7.1	7.0
60	10:09.5	10:16.4	10:23.2	10:29.9	10:36.6	10:43.2	10:49.7	10:56.2	11:02.6	11:08.9
	6.9	6.8	6.7	6.7	6.6	6.5	6.5	6.4	6.3	6.2
70	11:15.1	11:21.3	11:27.4	11:33.4	11:39.3	11:45.2	11:51.1	11:56.9	12:02.6	12:08.2
	6.2	6.1	6.0	5.9	5.9	5.9	5.8	5.7	5.6	5.6
80	12:13.8	12:19.3	12:24.6	12:29.8	12:34.9	12:39.9	12:44.8	12:49.6	12:54.3	12:58.9
	5.5	5.3	5.2	5.1	5.0	4.9	4.8	4.7	4.6	4.6
90	13:03.5	13:08.1	13:12.7	13:17.2	13:21.7	13:26.2	13:30.8	13:35.3	13:39.8	13:44.3
	4.6	4.6	4.5	4.5	4.5	4.6	4.5	4.5	4.5	4.6
100	13:48.9	13:53.4	13:57.9	14:02.3	14:06.8	14:11.2	14:15.7	14:20.1	14:24.6	14:29.0
	4.5	4.5	4.4	4.5	4.4	4.5	4.4	4.5	4.4	

SKP (along one path through the mantle longitudinal, along the other transverse; along the path through the outer core longitudinal) was reported by one station a few seconds earlier than calculated from the tables[8, 9] One $P'P'$ (longitudinal wave which has passed twice through the whole earth, travel time about 40 minutes) had a residual of -8 seconds from the Gutenberg-Richter tables[10] (corresponding to -4 seconds of P') and one *PKKP* (reflected inside the core from the core boundary) observed near a distance of 110° had a travel time exactly in agreement with the tables of Gutenberg-Richter.[11]

The errors of the average travel time curves for direct longitudinal waves are now probably smaller than the effects of the local structure near the source and the station. The corresponding differences in travel time depend mainly on the depth of the Mohorovičić discontinuity at the source (in shallow earthquakes) and the station and may amount to 3 seconds or even more at distances of a few degrees and at least 2 seconds at greater distances.

* Division of the Geological Sciences, Contribution No. 637.

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[6] Gutenberg, B., and Richter, C. F., *Gerlands Beitr. Geophys.*, **54**, 113 (1939).

[7] *Ibid.*, p. 115, table 19, column C.

[8] *Ibid.*, p. 126.

[9] Jeffreys, H., *Geophys. Suppl. Mon. Not. R. Astron. Soc.*, **4**, 609 (1939).

[10] Gutenberg, B., and Richter, C. F., *Gerlands Beitr. Geophys.*, **54**, 131 (1939).

[11] *Ibid.*, p. 127.

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*APPROXIMATE METHODS IN MATHEMATICS**

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Communicated by C. E. K. Mees, June 15, 1953

The following simple ideas have proved advantageous in solving systematically certain problems in geometrical optics and appear to be of general mathematical interest. The method is not restricted to power series approximations, but is applicable to any system of approximating functions, P_r, which have the quality that $P_i \cdot P_j$ can be approximately represented as a linear combination of polynomials P_r with r smaller than a constant n. However, in this note we shall restrict ourselves to polynomials.

In approximating functions by polynomials of one, two, or more variables, it is customary to neglect terms of degree greater than a given number n, so that the product of two polynomials of degree n again becomes a polynomial of degree n. In this case, the polynomials of degree n form an algebra with the unity as a module and the quantities $x^p y^q z^r \ldots$ as independent basis elements. The system is, in the language of algebra, a commutative, nilpotent system with unity added, since each of the other basis elements is a root of zero.

Power Series in One Variable.—Let us consider the function

$$f(x) = a_0 + a_1 x + a_{11} x^2 + \ldots a_{11} \ldots x^n. \qquad (1)$$

Taking the case $n = 5$, for example, and considering $1, x, x^2, \ldots x^5$ as the basis elements, we obtain the multiplication table (assuming $x^m = 0$ for $m > 5$)

	1	x	x^2	x^3	x^4	x^5
1	1	x	x^2	x^3	x^4	x^5
x	x	x^2	x^3	x^4	x^5	0
x^2	x^2	x^3	x^4	x^5	0	0
x^3	x^3	x^4	x^5	0	0	0
x^4	x^4	x^5	0	0	0	0
x^5	x^5	0	0	0	0	0

(2)

which shows that the corresponding algebra is commutative. The theory of hypercomplex numbers[1] shows that the matrix:

$$\begin{Vmatrix} a_0 & 0 & 0 & 0 & 0 & 0 \\ a_1 & a_0 & 0 & 0 & 0 & 0 \\ a_{11} & a_1 & a_0 & 0 & 0 & 0 \\ a_{111} & a_{11} & a_1 & a_0 & 0 & 0 \\ a_{1111} & a_{111} & a_{11} & a_1 & a_0 & 0 \\ a_{11111} & a_{1111} & a_{111} & a_{11} & a_1 & a_0 \end{Vmatrix} \qquad (3)$$

gives a regular representation of the function space, i.e., the product of two functions $f(x) = \sum_0^5 a_i x_i$, $g(x) = \sum_0^5 b_i x_i$ is a function $h(x) = \sum_0^5 c_i x_i$

such that the matrix (3) formed from the coefficients is the product of the matrices (3) formed from the a_i and b_i, respectively.

The reader may notice that here, and in the following examples, only the first column of a matrix of the form (3) has to be computed; the others are then already determined.

Power Series in Two Variables.—Let us take as the next example the polynomial with two variables

$$f = a_0 + a_1x + a_{11}x^2 + a_{111}x^3 + a_{1111}x^4 + y(a_2 + a_{12}x + a_{112}x^2 + a_{1112}x^3) + y^2(a_{22} + a_{122}x + a_{1122}x^2) + y^3(a_{222} + a_{1222}x) + y^4a_{2222}. \quad (4)$$

The multiplication table becomes

1	x	y	x^2	xy	y^2	x^3	x^2y	xy^2	y^3	x^4	x^3y	x^2y^2	xy^3	y^4
x	x^2	xy	x^3	x^2y	xy^2	x^4	x^3y	x^2y^2	xy^3					
y	xy	y^2	x^2y	xy^2	y^3	x^3y	x^2y^2	xy^3	y^4					
x^2	x^3	x^2y	x^4	x^3y	x^2y^2									
xy	x^2y	xy^2	x^3y	x^2y^2	xy^3									
y^2	xy^2	y^3	x^2y^2	xy^3	y^4									
x^3	x^4	x^3y												
x^2y	x^3y	x^2y^2												
xy^2	x^2y^2	xy^3												
y^3	xy^3	y^4												
x^4														
x^3y														
x^2y^2														
xy^3														
y^4														

(5)

in which the blank spaces in the matrices stand for zeros, and, according to the theory of hypercomplex numbers, the regular representation is given by the group matrix

a_0														
a_1	a_0													
a_2		a_0												
a_{11}	a_1		a_0											
a_{12}	a_2	a_1		a_0										
a_{22}		a_2			a_0									
a_{111}	a_{11}		a_1			a_0								
a_{112}	a_{12}	a_{11}	a_2	a_1			a_0							
a_{122}	a_{22}	a_{12}		a_2	a_1			a_0						
a_{222}		a_{22}			a_2				a_0					
a_{1111}	a_{111}		a_{11}			a_1				a_0				
a_{1112}	a_{112}	a_{111}	a_{12}	a_{11}		a_2	a_1				a_0			
a_{1122}	a_{122}	a_{112}	a_{22}	a_{12}	a_{11}		a_2	a_1				a_0		
a_{1222}	a_{222}	a_{122}		a_{22}	a_{12}			a_2	a_1				a_0	
a_{2222}		a_{222}			a_{22}				a_2					a_0

(6)

Three Variables.—Finally we shall investigate three variables to show the typical form for the parastrophic matrix as well as the group matrix.

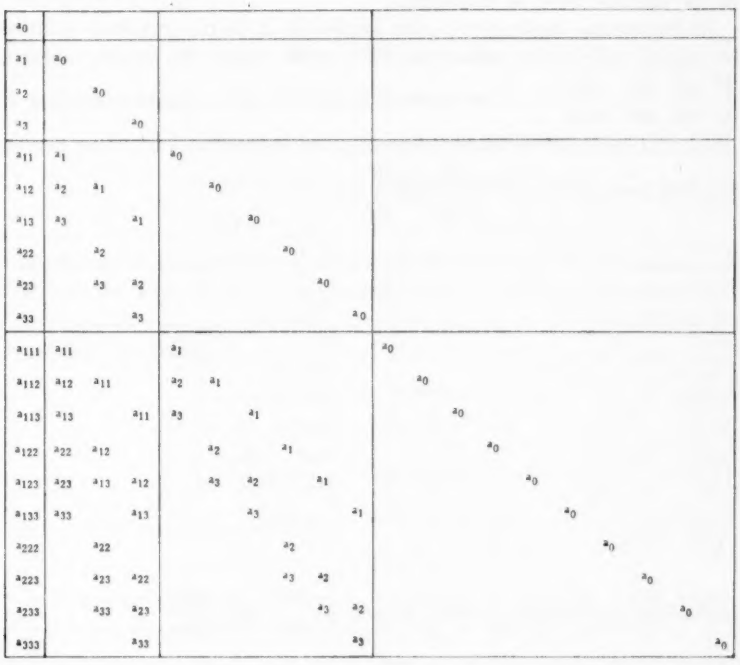
$$f(x, y, z) = a_0 + (a_1x + a_2y + a_3z) + (a_{11}x^2 + a_{12}xy + a_{12}xz + a_{22}y^2 + a_{23}yz + a_{33}z^2) + (a_{111}x^3 + a_{112}x^2y + a_{113}x^2z + a_{122}xy^2 + a_{123}xyz + a_{133}xz^2 + a_{222}y^3 + a_{223}y^2z + a_{233}yz^2 + a_{333}z^3). \quad (7)$$

We find as multiplication table

1	x	y	z	x^2	xy	xz	y^2	yz	z^2	x^3	x^2y	x^2z	xy^2	xyz	xz^2	y^3	y^2z	yz^2	z^3
x	x^2	xy	xz	x^3	x^2y	x^2z	xy^2	xyz	xz^2										
y	xy	y^2	yz	x^2y	xy^2	xyz	y^3	y^2z	yz^2										
z	xz	yz	z^2	x^2z	xyz	xz^2	y^2z	yz^2	z^3										
x^2	x^3	x^2y	x^2z																
xy	x^2y	xy^2	xyz																
xz	x^2z	xyz	xz^2																
y^2	xy^2	y^3	y^2z																
yz	xyz	y^2z	yz^2																
z^2	xz^2	yz^2	z^3																
x^3																			
x^2y																			
x^2z																			
xy^2																			
xyz																			
xz^2																			
y^3																			
y^2z																			
yz^2																			
z^3																			

(8)

with the group matrix

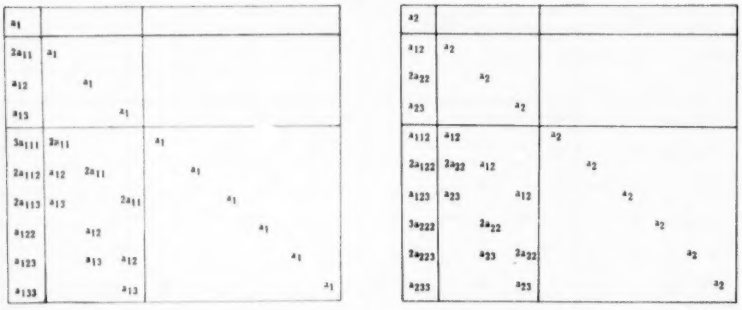


(9)

We have proved, for any polynomial function, the existence of a corresponding triangular matrix (containing in its first column the coefficients of the polynomials) such that the sum or product of two functions is obtained from the sum or product of the two corresponding matrices. This makes it possible to transform any equation between polynomials into equations between triangular matrices and thus to solve by an iteration

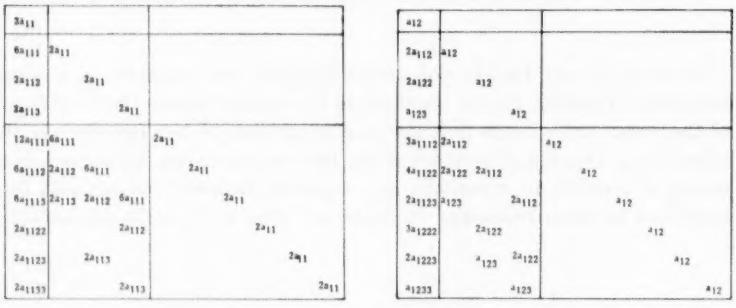
procedure any problem where the polynomial approximation is permissible in the neighborhood of the origin.

An important application of the method is in the approximate solution of partial differential equations. We write down the group matrices $\frac{\partial f}{\partial x}, \frac{\partial f}{\partial y}, \frac{\partial^2 f}{\partial x^2}, \frac{\partial^2 f}{\partial x \partial y}, \ldots$, for the function (7) of three variables, keeping in mind that each differentiation decreases the approximation by one degree. We find the matrices, A, for $\frac{\partial f}{\partial x}$ and $\frac{\partial f}{\partial y}$ as



(10)

and going to the fourth power, we find for $\frac{\partial^2 f}{\partial x^2}$ and $\frac{\partial^2 f}{\partial x \partial y}$



(11)

which suffice to show the general law. Let us give some examples which show how easily partial differential equations are solved by this method.

To find a function $f(x, y)$ so that $p = \frac{\partial f}{\partial x}$ and $q = \frac{\partial f}{\partial y}$ obey the equation

$$p^2 + q^2 = 1 \tag{12}$$

we calculate with the matrices (10) and obtain the successive equations

$$\begin{aligned} a_1^2 + a_2^2 &= 1 \\ 4a_1a_{11} + 2a_2a_{12} &= 0 \\ 2a_1a_{12} + 4a_2a_{22} &= 0 \\ 6a_1a_{111} + 4a_{11}^2 + 2a_2a_{112} + a_{12}^2 &= 0 \\ 4a_1a_{112} + 4a_{11}a_{12} + 4a_2a_{122} + 4a_{22}a_{12} &= 0 \\ 2a_1a_{122} + a_{12}^2 + 6a_2a_{222} + 4a_{22}^2 &= 0. \end{aligned} \tag{13}$$

The reader will notice that, if we are given a_1, a_{11}, a_{111} our coefficients, with the exception of a_2, can be computed linearly. This is a common occurrence in this method. Having chosen for a_2 one of the roots of the first equation, we find

$$\begin{aligned} a_2a_{12} &= -2a_1a_{11} \\ a_2^2a_{22} &= a_1^2a_{11} \\ a_2^3a_{112} &= -(3a_1a_2^2a_{111} + 2a_{11}^2) \\ a_2^4a_{122} &= a_1(3a_1a_2^2a_{111} + 4a_{11}^2) \\ a_2^5a_{222} &= -a_1^2(a_1a_2^2a_{111} + 2a_{11}^2). \end{aligned} \tag{14}$$

As another example, we choose a differential equation which plays a certain role in optics and is hard to attack by classical methods

$$(p^2 + q^2)y = q. \tag{15}$$

The calculation is simplified by concluding that q vanishes for $y = 0$. This means that the coefficients

$$a_2 = a_{12} = a_{112} = a_{1112} = a_{11112} = 0. \tag{16}$$

We find from the matrix calculation the recursion formulae

$$\begin{aligned} 2a_{22} &= a_1^2 & 2a_{1122} &= 6a_1a_{111} + 4a_{11}^2 \\ 2a_{122} &= 4a_1a_{11} & 3a_{1222} &= 0 \\ 3a_{222} &= 0 & 4a_{2222} &= 2a_1a_{122} + 4a_{22}^2 \end{aligned} \tag{17}$$

which determine all coefficients, if a_1, a_{11}, a_{111}, a_{1111} are given. Up to the fourth order we find thus

$$\begin{aligned} a_2 &= 0 & a_{12} &= 0 & a_{22} &= (1/2)a_1^2 \\ a_{112} &= 0 & a_{122} &= 2a_1a_{11} & a_{222} &= 0 \\ a_{1112} &= 0 & a_{1122} &= 3a_1a_{111} + 2a_{11}^2 & a_{1222} &= 0 \\ & & & & a_{2222} &= (1/4)a_1^4 + a_1^2a_{11}. \end{aligned} \tag{18}$$

The method described here gives the development of the solution of the partial differential equation for the neighborhood of $x = y = 0$. It can break down only if the zero point is a singular point.

Of course there is, in general, no difficulty in obtaining the approximate solution for a series around an *arbitrary* point. In boundary problems, there remains the question of fitting the undetermined coefficients (a_1, a_{11}, ...) to the boundary values.

The method having been established, the reader will find no difficulty in applying it to the more common way of writing a polynomial,

$$f(x, y) = b_0 + b_1x + b_2y + (1/2)(b_{11}x^2 + 2b_{12}xy + b_{22}y^2) + (1/6)(b_{111}x^3 + 3b_{112}x^2y + 3b_{122}xy^2 + b_{222}y^3)\ldots, \quad (19)$$

or to the problem of developing functions of several variables, instead of into a power series, into a series of other functions, having the quality that

$$A_\nu \cdot A_\mu = \sum^{\tau < r\mu} \alpha_{ik}^{\lambda} A_\lambda, \quad (20)$$

where every product is a linear combination of the functions with index no greater than that of the highest of the original functions.

* Communication No. 1589 from the Kodak Research Laboratories.

[1] Cf. the matrices S_i appearing in Frobenius, G., *Theorie der hyperkomplexen Grossen*, Berl. Sitzungsber., 1903, and Herzberger, M., *Ueber Systems hyperkomplexer Grossen*, Inaugural Dissertation, Berlin, 1923.

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*ERGODIC THEORY OF MARKOV CHAINS ADMITTING AN INFINITE INVARIANT MEASURE**

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1. Introduction.—We consider real valued Markov chains ..., x_{-1} x_0, x_1, ... with a stationary transition probability function

$$H(u, B) = \text{Prob}\,(x_{n+1} \in B \mid x_n = u).$$

Under certain conditions there exists a probability measure π defined on the Borel sets B and satisfying

$$\pi(B) = \int \pi(du)H(u, B). \quad (1)$$

There is then a stationary Markov chain ... x_{-1}, x_0, x_1, ... with the transition function H such that Prob $(x_n \in B) = \pi(B)$, $n = 0 \pm 1, \ldots$. Birkhoff's ergodic theorem can then be applied to this chain (cf. ref. 2).

It may be that no probability measure π exists satisfying (1). (Such is the case, for example, if for every bounded Borel set B, $\lim_{k\to\infty} H_k(u, B) = 0$, where H_k denotes the k-step transition function, $H_1 = H$.) There may, however, be an infinite measure π which satisfies (1). The purpose of this note is to show how the ergodic theorem of Hopf[3] can then be applied. A similar treatment will be given elsewhere for certain stochastic processes involving a continuous time parameter (cf. ref. 5). The present note is closely related to recent work of Robbins[7] and of Kallianpur and Robbins.[4]

2. Definition of the Measure m.—We shall assume henceforth that (1) *has an "admissible" solution* π*;* i.e., π is a measure on the real Borel sets which satisfies (1), does not vanish identically, and is finite for *bounded* Borel sets. (Current unpublished work of C. Derman shows that this is so in a variety of circumstances.)

Let F_0 be the class of real Borel sets, Ω the space of sequences x of real numbers, $x = (\ldots, x_{-1}, x_0, x_1, \ldots)$, and F the Borel extension of the cylinder sets in Ω. If $A \in F$ is determined by the coordinates x_k, x_{k+1}, $\ldots$, then $Q(A \mid x_k = u)$ will denote the probability of A relative to the Markov chain starting with $x_k = u$, as specified by H.

A measure m may be set up on F as follows. If A is a cylinder set determined by $x_k, \ldots, x_r$, let

$$m(A) = \int \pi(du) Q(A \mid x_j = u), \tag{2}$$

where (1) ensures that any value of $j \leq k$ may be used indifferently in (2). It can be verified that m may be extended to F, there being no essential change in the proof of the extension theorem of Kolmogorov.[6] The extended measure m satisfies (2) whenever $A \in F$ depends on x_k, x_{k+1}, $\ldots$ and $j \leq k$. Moreover, $m(A) = 0$ if and only if $Q(A \mid x_j = u) = 0$ for a.e. (π) u; thus $m(A) = 0$ has a meaning in terms of ordinary Markov chain probabilities. From (1) it follows that if $A \in F$ and if T is the shift transformation, $(Tx)_t = x_{t+1}$, then $m(A) = m(TA)$.

3. The Dissipative Part of Ω.—For any $B \in F_0$, let R_B be the event that $x_n \in B$ infinitely often for $n = 1, 2, \ldots$ ("$x_n \in B$ i.o.").

ASSUMPTION 1. *If* $B \in F_0$ *then* $Q(R_B \mid x_0 = u) = 1$ *for* a.e. (π) u *in* B.

THEOREM 1. *If Assumption 1 holds then the dissipative part of* Ω *has m-measure 0 (see ref. 3 for terminology).*

Sketch of Proof: Let A be a cylinder set defined by, say, $(x_0, x_1, \ldots, x_k) \in B_{k+1}$, a $(k+1)$-dimensional Borel set, and let

$$U_n = \left\{u; \frac{1}{n+1} < Q(A \mid x_0 = u) \leq \frac{1}{n}\right\}, \qquad n = 1, 2, \ldots.$$

Equation (2) ensures that A differs from $\sum_{n=1}^{\infty} A \cdot (x_0 \in U_n)$ by a set of

m-measure 0, and hence for a.e. $(m)x$ in A, $T^n x \in A$ i.o., so that $m(A \cdot \sum_1^\infty T^{-n}A) = m(A)$. Next, let W be a "wandering" set with $m(W) < \infty$ (it suffices to consider this case) and let A be a cylinder set such that $m(W - A) + m(A - W) < \epsilon$. Then (all sums $n = 1$ to ∞)

$$0 = m(W \cdot \sum T^n W) > m(A \cdot \sum T^n W) - \epsilon$$
$$= \sum m[(T^{-n}A) \cdot W] - \epsilon \geq m(W \cdot \sum T^{-n}A) - \epsilon >$$
$$m(A \cdot \sum T^{-n}A) - 2\epsilon = m(A) - 2\epsilon,$$

and hence

$$m(W) \leq m(A) + \epsilon < 3\epsilon, \qquad m(W) = 0.$$

4. Metric Transitivity of T.—The following assumption is stronger than Assumption 1.

ASSUMPTION 2. *If $B \in F_0$ and $\pi(B) > 0$ then $Q(R_B | x_0 = u) = 1$ for* a.e. (π) u *in* Ω.

THEOREM 2. *If Assumption 2 holds then the shift transformation T is metrically transitive.*

Sketch of Proof: First, let $A \in F$ be such that for every $k = 1, 2, \ldots$, A depends only on $x_k, x_{k+1}, \ldots$ (call such a set a "terminal" set), and suppose $A = TA$. Assumption 2 and the invariance of A can be shown to imply that $Q(A | x_0 = u)$ is constant for a.e. $(\pi)u$, and the 0–1 law shows that the constant is 0 or 1. It follows from (2) that $m(A) = 0$ or $m(\Omega - A) = 0$.

Now let $g(x)$ be a fixed positive m-summable function which depends on a finite number of coordinates of x. Hopf's theorem implies that if $f(x)$ is m-summable then

$$\lim_{n \to \infty} \frac{\sum_0^n f(T^j x)}{\sum_0^n g(T^j x)} = \mu(f; x)$$

exists for a.e. $(m)x$. If $f(x)$ depends on only a finite number of coordinates it follows from the result above on "terminal" invariant sets that

$$\mu(f; x) = \text{const.} = \frac{\int f(x) m(dx)}{\int g(x) m(dx)}. \tag{3}$$

To prove metric transitivity it is sufficient to show that (3) holds for every m-summable f.

Now let $f(x)$ be any m-summable function and let $f_k(x)$ be a sequence of functions of a finite number of coordinates such that

$$\lim_{k \to \infty} \int |f(x) - f_k(x)| m(dx) = 0.$$

Then

$$\mu(f;\ x) = \mu(f - f_k;\ x) + \frac{\int f_k(x)m(dx)}{\int g(x)m(dx)},$$

whence

$$\left|\mu(f;\ x) - \frac{\int f(x)m(dx)}{\int g(x)m(dx)}\right| = \lim_{k\to\infty} \left|\mu(f - f_k;\ x)\right|.$$

Since for any m-summable $h(x)$, $\int \mu(h;\ x)g(x)m(dx) = \int h(x)m(dx)$, it follows that

$$\lim_{k\to\infty} \int \left|\mu(f - f_k;\ x)\right| g(x)m(dx) \leq \lim_{k\to\infty} \int \mu(|f - f_k|;\ x)g(x)m(dx) = \lim_{k\to 0} \int \left||f - f_k|\right| m(dx) = 0,$$

and since $g(x) > 0$ the desired result follows.

5. *Applications.*—In what follows $h(u)$ and $k(u)$ will be π-summable functions of a real variable u with $\int k(u)\pi(du) \neq 0$. Theorems 1 and 2 in conjunction with Hopf's theorem then imply

THEOREM 3.[8] *Under Assumption 2, for a.e.* $(\pi)x_0$,

$$\text{Prob}\left\{\lim_{n\to\infty} \frac{h(x_0) + \ldots + h(x_n)}{k(x_0) + \ldots + k(x_n)} = \frac{\int_{-\infty}^{\infty} h(u)\pi(du)}{\int_{-\infty}^{\infty} k(u)\pi(du)}\right\} = 1.$$

COROLLARY. *Under Assumption 2, if* π_1 *is another admissible solution of (1) such that* $\pi(B) = 0$ *implies* $\pi_1(B) = 0$, *then for some constant* $c > 0$, $\pi_1 = c\pi$.

Next, consider a sequence of independent random variables $y_1, y_2, \ldots$ with a common distribution function $F(y)$.

ASSUMPTION 3. *The sums* $y_1 + \ldots + y_n$ *are "interval-recurrent"; i.e., for any interval I, Prob* $(y_1 + \ldots + y_n \in I \text{ i.o.}) = 1$ *(see ref. 1).*

We state without proof

LEMMA 1. *Under Assumption 3, if* $B \in F_0$ *has positive Lebesgue measure then for a.e. a, Prob*$(a + y_1 + \ldots + y_n \in B \text{ i.o.}) = 1$. *This is true for every a if some convolution* $F^{n*}(y)$ *has an absolutely continuous component.*

Set $H(u, B) = \int_B dF(y - u)$; then π = Lebesgue measure satisfies (1), and from Theorem 3 and Lemma 1 we obtain

THEOREM 4. *Under Assumption 3, for a.e. (Lebesgue measure) a,*

$$\text{Prob}\left\{\lim_{n\to\infty} \frac{\sum_{j=1}^{n} h(a + y_1 + \ldots + y_j)}{\sum_{j=1}^{n} k(a + y_1 + \ldots + y_j)} = \frac{\int_{-\infty}^{\infty} h(u)du}{\int_{-\infty}^{\infty} k(u)du}\right\} = 1. \quad (4)$$

If some convolution $F^{n*}(y)$ has an absolutely continuous component then (4) holds for *every* a.

From Theorem 4 we can deduce the following

COROLLARY. *Under Assumption 3 (even if no $F^{n*}(y)$ has an absolutely continuous component) for every a the limit relation in (4) holds with probability 1 simultaneously for every two functions $h(u)$, $k(u)$ which are Riemann-integrable in a finite interval and vanish elsewhere, the integral of $k(u)$ assumed to be $\neq 0$.*

Thus the prob 1 *density* of the sequence of sums $y_1 + \ldots + y_n$ implies the prob 1 *equidistribution* of these sums. This establishes a conjecture made in ref. 4.

If the y_i have only integer values then the function $\pi(B)$ = number of integer points in B, is an admissible solution of (1). Theorem 3 then implies that under Assumption 2 (which holds whenever every integer ν is a "recurrent" value of the sums $y_1 + \ldots + y_n$), for every two functions of integers $h(\nu)$, $k(\nu)$ such that

$$\sum_{-\infty}^{\infty} |h(\nu)| < \infty, \sum_{-\infty}^{\infty} |k(\nu)| < \infty, \sum_{-\infty}^{\infty} k(\nu) \neq 0,$$

$$\text{Prob}\left\{ \lim_{n\to\infty} \frac{\sum_{j=1}^{n} h(y_1 + \ldots + y_j)}{\sum_{j=1}^{n} k(y_1 + \ldots y_j)} = \frac{\sum_{-\infty}^{\infty} h(\nu)}{\sum_{-\infty}^{\infty} k(\nu)} \right\} = 1. \qquad (5)$$

* Work sponsored by the Office of Scientific Research of the Air Force.

† On leave from the RAND Corporation.

[1] Chung, K. L., and Fuchs, W. H. J., "On the Distribution of Values of Sums of Random Variables," *Mem. Am. Math. Soc.*, **6**, (1951).

[2] Doob, J. L., *Stochastic Processes*, Wiley & Son, New York, 1953, Chap. 10.

[3] Hopf, E., *Ergodentheorie*, reprinted by Chelsea, New York, 1948, pp. 46–53.

[4] Kallianpur, G., and Robbins, H., "On the Sequence of Partial Sums of Independent Random Variables," to appear.

[5] Kallianpur, G., and Robbins, H., "Ergodic Property of the Brownian Motion Process"; to appear in PROC. NATL. ACAD. SCI.

[6] Kolmogorov, A., *Grundbegriffe der Warscheinlichkeitsrechnung*, reprinted by Chelsea, New York, 1946, pp. 24–30.

[7] Robbins, H., "On the Equidistribution of Sums of Independent Random Variables"; to appear in *Proc. Am. Math. Soc.*

[8] Chung has obtained this result independently and by a different method for the case in which the X_i have only a denumerable set of values. His results will appear in *Trans. Am. Math. Soc.*

ON COHOMOLOGY GROUPS OF COMPACT ANALYTIC VARIETIES WITH COEFFICIENTS IN SOME ANALYTIC FAISCEAUX*

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1. Introduction.—Let V be a compact complex analytic variety of complex dimension n and let F be an analytic fibre bundle over V whose fibre is a complex line, i.e., the complex number field C and whose structure group is the multiplicative group C^* of complex numbers acting on C. Moreover let $\Omega^p(F)$ be the faisceau[1] over V of germs of holomorphic p-forms with coefficients in F. The faisceau $\Omega^p(F)$ introduced recently by D. C. Spencer and, independently by J.-P. Serre turned out to be of importance to applications of faisceaux to the theory of compact analytic varieties. However, for those applications, we need a basic theorem to the effect that the cohomology groups $H^q(V;\ \Omega^p(F))$ of V with coefficients in $\Omega^p(F)$ have finite dimension. The purpose of the present short note is to give an outline of a proof of this basic theorem.

2. Harmonic Forms with Coefficients in Complex Line Bundles.—Take a sufficiently fine finite covering $\mathfrak{U} = \{U_j\}$ of V. Then, letting π be the projection of F onto V, $\pi^{-1}(U_j)$ is a direct product: $\pi^{-1}(U_j) = C \times U_j$ and $\zeta_j \times z \in C \times U_j$ is identical with $\zeta_k \times z \in C \times U_k$ if and only if $\zeta_j = f_{jk}(z)\zeta_k$, where $f_{jk}(z)$ is a non-vanishing holomorphic function defined in $U_j \cap U_k$. Under these circumstances we say that *the bundle F is defined by the system of functions* $\{f_{jk}\}$.

Setting $a_{jk} = |f_{jk}|^2$, we infer that the system of functions $\{a_{jk}\}$ defines a principal bundle over V of class C^∞ whose structure group is the multiplicative group of positive real numbers. Obviously this bundle is topologically trivial and therefore we can find a system $\{a_j\}$ of positive real functions a_j of class C^∞ defined, respectively, in U_j such that

$$|f_{jk}|^2 = a_j/a_k, \qquad \text{in } U_j \cap U_k.$$

By a form φ of type (p, q) of class C^∞ with coefficients in F we shall mean a system $\{\varphi_j\}$ of exterior differential forms φ_j of type (p, q) of class C^∞ defined, respectively, in U_j such that

$$\varphi_j = f_{jk} \cdot \varphi_k, \qquad \text{in } U_j \cap U_k.$$

We denote by $\Gamma^{p,q}(F)$ the linear space consisting of all forms φ of type (p, q) of class C^∞ with coefficients in F. We introduce an arbitrary but fixed positive definite Hermitian metric $ds^2 = 2\sum g_{\alpha\bar{\beta}}\,(dz^\alpha d\bar{z}^\beta)$ on V and denote by $*\varphi_j$ the adjoint form of φ_j with respect to this metric ds^2.

Now, for any $\varphi = \{\varphi_j\} \in \Gamma^{p,q}(F)$, we define $\bar{\partial}\varphi = \{(\bar{\partial}\varphi)_j\}$ and $\mathfrak{d}\varphi = \{(\mathfrak{d}\varphi)_j\}$ by

$$(\bar{\partial}\varphi)_j = \bar{\partial}\varphi_j,$$

$$(\mathfrak{d}\varphi)_j = -*a_j\partial\left(\frac{1}{a_j}*\varphi_j\right),$$

respectively, where ∂ or $\bar{\partial}$ denotes the exterior derivation with respect to the variables z^α or $\bar{z}^\alpha$. As one readily infers, $\bar{\partial}\varphi$ and $\mathfrak{d}\varphi$ have invariant meaning, i.e., we have $(\bar{\partial}\varphi)_j = f_{jk}\cdot(\bar{\partial}\varphi)_k$ and $(\mathfrak{d}\varphi)_j = f_{jk}\cdot(\mathfrak{d}\varphi)_k$. Therefore, $\bar{\partial}\varphi \in \Gamma^{p,q+1}(F)$ and $\mathfrak{d}\varphi \in \Gamma^{p,q-1}(F)$ for any $\varphi \in \Gamma^{p,q}(F)$. Next, for any pair of forms $\varphi, \psi \in \Gamma^{p,q}(F)$, we define the inner product (φ, ψ) by

$$(\varphi, \psi) = \int_V \frac{1}{a_j}\varphi_j \wedge *\bar{\psi}_j,$$

where we note that

$$\frac{1}{a_j}\varphi_j \wedge *\bar{\psi}_j = \frac{1}{a_k}\varphi_k \wedge *\bar{\psi}_k$$

in $U_j \cap U_k$. The linear operator $\mathfrak{d}$ is the formal adjoint of $\bar{\partial}$ in the sense that

$$(\varphi, \mathfrak{d}\psi) = (\bar{\partial}\varphi, \psi) \tag{1}$$

for arbitrary form $\varphi \in \Gamma^{p,q}(F)$, $\psi \in \Gamma^{p,q+1}(F)$. In fact, this formula (1) follows from the identity

$$d\left(\frac{1}{a_j}\varphi_j \wedge *\bar{\psi}_j\right) = \frac{1}{a_j}\partial\varphi_j \wedge *\bar{\psi}_j - \frac{1}{a_j}\varphi_j \wedge *(\mathfrak{d}\bar{\psi})_j.$$

Finally we introduce the *complex Laplace-Beltrami operator*[2]

$$\square = \mathfrak{d}\bar{\partial} + \bar{\partial}\mathfrak{d}.$$

Then we have, for arbitrary forms $\varphi, \psi \in \Gamma^{p,q}(F)$,

$$(\square\varphi, \psi) = (\varphi, \square\psi) = (\bar{\partial}\varphi, \bar{\partial}\psi) + (\mathfrak{d}\varphi, \mathfrak{d}\psi). \tag{2}$$

Moreover we infer readily that the *principal part* of the differential operator $\square$ is $-\Sigma g^{\alpha\bar{\beta}}\dfrac{\partial^2}{\partial z^\alpha \partial \bar{z}^\beta}$, that is,

$$\square = -\sum g^{\alpha\bar{\beta}}\frac{\partial^2}{\partial z^\alpha \partial \bar{z}^\beta} + \ldots,$$

where $\ldots$ denotes the terms containing at most the first partial derivatives. Thus $\square$ is an elliptic partial differential operator of the second order.

The theory of harmonic forms—that is the theory of differential operators, d, δ and $\Delta = \delta d + d\delta$—can be easily generalized to the differential operators, $\bar{\partial}$, $\mathfrak{d}$ and $\square = \mathfrak{d}\bar{\partial} + \bar{\partial}\mathfrak{d}$. In particular, letting $H^{p,q}(F)$ be the subspace of $\Gamma^{p,q}(F)$ consisting of all solutions φ of the Laplace-Beltrami equation $\square\varphi = 0$, we have the *decomposition formula*[3]

$$\Gamma^{p,q}(F) = \bar{\partial}\Gamma^{p,q-1}(F) \oplus \mathfrak{d}\Gamma^{p,q+1}(F) \oplus H^{p,q}(F), \tag{3}$$

where $\oplus$ denotes a direct sum of mutually orthogonal linear subspaces. Since the equation $\square\varphi = 0$ is equivalent to $\bar{\partial}\varphi = \mathfrak{d}\varphi = 0$, as (2) shows, *$H^{p,q}(F)$ is the linear space consisting of all forms φ of type (p, q) of class C^∞ with coefficients in Γ satisfying $\bar{\partial}\varphi = \mathfrak{d}\varphi = 0$. We call such φ a harmonic form of type (p, q) with coefficients in F. Now, since* $\square$ is a partial differential operator of *elliptic type* with the typical principal part $-\Sigma g^{\alpha\bar{\beta}} \dfrac{\partial^2}{\partial z^\alpha \partial \bar{z}^\beta}$ defined over the *compact* variety V, we infer that the number of linearly independent solutions φ of the equation $\square\varphi = 0$ is finite. Thus we obtain

$$\dim H^{p,q}(F) < +\infty. \tag{4}$$

3. Cohomology Groups with Coefficients in $\Omega^p(F)$.—Let $\Omega^p(F)$ be the faisceau over V of germs of holomorphic p-forms with coefficients in F and let $H^q(V; \Omega^p(F))$ be the qth cohomology group of V with coefficients in $\Omega^p(F)$. In the special case in which F is the trivial bundle $C \times V$, P. Dolbeault[4] has proved that $H^q(V; \Omega^p(C \times V))$ is isomorphic to the $\bar{\partial}$-cohomology of forms of type (p, q) on V. This result of Dolbeault can be easily generalized to our general case, as was pointed out by J.-P. Serre. Namely we have the isomorphism

$$H^q(V; \Omega^p(F)) \cong Z^{p,q}(F)/\bar{\partial}\Gamma^{p,q-1}(F), \tag{5}$$

where $Z^{p,q}(F)$ is the subspace of $\Gamma^{p,q}(F)$ consisting of all $\varphi \in \Gamma^{p,q}(F)$ satisfying $\bar{\partial}\varphi = 0$.

Now we infer from the decomposition formula (3) that

$$Z^{p,q}(F) = \bar{\partial}\Gamma^{p,q-1}(F) \oplus H^{p,q}(F).$$

This proves the isomorphism

$$Z^{p,q}(F)/\bar{\partial}\Gamma^{p,q-1}(F) \cong H^{p,q}(F).$$

Combining this with (5), we obtain

$$H^q(V; \Omega^p(F)) \cong H^{p,q}(F).$$

Therefore, by (4), the cohomology group $H^q(V; \Omega^p(F))$ has a finite dimension. Thus we have proved the following

THEOREM. *The qth cohomology group $H^q(V; \Omega^p(F))$ of a compact analytic variety V with coefficients in the analytic faisceau $\Omega^p(F)$ is isomorphic to the*

linear space $H^{p,q}(F)$ of all harmonic forms of type (p, q) on V with coefficients in F. In particular, the dimension of $H^q(V; \Omega^p(F))$ is always finite.

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[1] For the notion of analytic faisceau, see Séminaire, H. Cartan, 1951–1952.

[2] In the case where the bundle F is trivial, the operator $\square$ has been studied in detail by Garabedian, P. R., and Spencer, D. C., "A Complex Tensor Calculus for Kähler Manifolds," Technical Report 17, Stanford University (1951).

[3] Cf. Kodaira, K., "Harmonic Fields in Riemannian Manifolds," *Ann. Math.*, **50**, 587–665 (1949), Sections 4 and 10.

[4] Dolbeault, P., "Sur la cohomologie des variétés analytiques complexes," *Compt. rend., Paris*, **236**, 175–177 (1953).

GROUPS OF COMPLEX LINE BUNDLES OVER COMPACT KÄHLER VARIETIES*

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1. Introduction.—This is a preliminary report of a generalization of divisor class groups on algebraic varieties to groups of complex line bundles over compact Kähler varieties. In the case of algebraic varieties, a divisor class defines a complex line bundle whose structure group is the multiplicative group of complex numbers. Therefore, on Kähler varieties, we replace the notion of divisor classes by that of complex line bundles, and determine the structure of the group of such complex line bundles. In this way we are led to the definition of Picard varieties attached to Kähler varieties.

2. Groups of Complex Line Bundles.—We denote by C the complex line (i.e., the field of all complex numbers) and by C^* the multiplicative group of complex numbers acting on C. Let V be a compact Kähler variety of complex dimension n and let F be an analytic fibre bundle over V with the fibre C and the group C^*. In what follows we call such a bundle F simply a *complex line bundle*. Letting $\mathfrak{U} = \{U_j\}$ be a sufficiently fine finite covering of V and p be the projection of F onto V, we have $p^{-1}(U_j) = C \times U_j$, and $\zeta_j \times z \in C \times U_j$ is identical with $\zeta_k \times z \in C \times U_k$ if and only if $\zeta_j = f_{jk}(z)\cdot\zeta_k$, $f_{jk}(z)$ being a non-vanishing holomorphic function defined in $U_j \cap U_k$. We say that *the bundle F is defined by the system of functions f_{jk}*. Clearly we have

$$f_{jk}f_{kj} = 1, \qquad f_{jk}f_{kl}f_{lj} = 1. \tag{1}$$

Two bundles F, F' defined, respectively, by f_{jk}, f'_{jk} are said to be *analytically equivalent* if there exist non-vanishing holomorphic functions f_j defined,

respectively, in U_j such that $f'_{jk} = f_j f_k^{-1} f_{jk}$. Then we *identify F' with F and consider F' the same as F.*

Now, for any pair of bundles F, G determined, respectively, by f_{jk}, g_{jk}, we define $F \pm G$ to be the bundle determined by $f_{jk} g_{jk}^{\pm 1}$. Then the set $\mathfrak{F} = \{F\}$ of all complex line bundles can be regarded as an additive group. The zero element of the additive group $\mathfrak{F}$ is the trivial bundle $C \times V$.

Given a divisor D on V defined in each U_j by a local meromorphic function $R_j(D) = R_j(z; D)$, the system of functions $f_{jk}(D) = R_j(D)/R_k(D)$ defines a complex line bundle over V which will be denoted by $\{D\}$. It is obvious that $\{D\}$ coincides with $\{D'\}$ if and only if D is linearly equivalent to D'. Thus *each $\{D\}$ may be identified with the divisor class of D and therefore the divisor class group can be regarded as a subgroup of $\mathfrak{F}$.*

3. Characteristic Classes.—It follows from (1) that, for each triple (j, k, l),

$$\log f_{jk} + \log f_{kl} + \log f_{lj} = 2\pi i c_{jkl} \qquad (2)$$

is a constant in $U_j \cap U_k \cap U_l$ and that c_{jkl} is a rational integer. The system of these integers c_{jkl} defines a 2-cocycle on the nerve N of the covering $\mathfrak{U}$ and therefore determines an element c_N of the 2nd cohomology group $H^2(N; Z)$ with integer coefficients Z. Moreover, if $\mathfrak{U}' < \mathfrak{U}$ in the sense that each member $U'_j \in \mathfrak{U}'$ is contained in some $U_k \in \mathfrak{U}$, then the canonical homomorphism $H^2(N; Z) \to H^2(N'; Z)$ maps c_N onto $c_{N'}$; thus $c = \lim_N c_N$ is a well-defined element of the 2nd cohomology group $H^2(V; Z)$ of the variety V, where $\lim_N$ denotes the direct limit with respect to the partial order $<$. This c is called the *characteristic class*[1] of the bundle F. We denote the characteristic class c of F by $c(F)$. Clearly the mapping $F \to c(F)$ is a homomorphism of the group $\mathfrak{F}$ into $H^2(V; Z)$.

Now we shall show that, *for given $c \in H^2(V, Z)$, there exists a complex line bundle F with $c(F) = c$ if and only if the harmonic part Hc of the cohomology class c is of type (1.1).* Let $A^{q,p}$ be the faisceau over V of germs of forms of class C^∞ of type (q, p), $\Xi^{q,p}$ the subfaisceau of $A^{q,p}$ consisting of germs of forms closed under $\bar{\partial}$. Again, let B^r be the faisceau over V of germs of r-forms of Class C^∞ and Φ^r be the subfaisceau of germs of r-forms closed under d. Clearly $\Omega^q = \Xi^{q,0}$ is the faisceau of germs of holomorphic q-forms and $C = \Phi^0$ is the faisceau of complex constants.[2] We have the diagram

$$\begin{array}{ccccccccc}
0 & \longrightarrow & \Phi^p & \xrightarrow{i} & B^p & \xrightarrow{d} & \Phi^{p+1} & \longrightarrow & 0 \\
 & & \| & & \| & & \| & & \\
0 & \longrightarrow & \Xi^{0,p} & \xrightarrow{i} & A^{0,p} & \xrightarrow{\bar{\partial}} & \Xi^{0,p+1} & \longrightarrow & 0
\end{array}$$

where the two horizontal lines are exact, where $\Pi = \Pi_{0,p}$ is the canonical projection of B^p onto $A^{0,p}$, and i denotes the inclusion map. We obviously have commutativity, that is, $i\Pi_{0,p} = \Pi_{0,p}i$, $\bar{\partial}\Pi_{0,p} = \Pi_{0,p+1}d$. In particular, for $p = 0$, we have

$$\begin{array}{ccccccccc} 0 & \longrightarrow & C & \xrightarrow{i} & B^0 & \xrightarrow{d} & \Phi^1 & \longrightarrow & 0 \\ & & \downarrow{\scriptstyle i} & & \downarrow{\scriptstyle \Pi} & & \downarrow{\scriptstyle \Pi} & & \\ 0 & \longrightarrow & \Omega^0 & \xrightarrow{i} & A^{0,0} & \xrightarrow{\bar{\partial}} & \Xi^{0,1} & \longrightarrow & 0 \end{array}$$

From the corresponding exact cohomology sequences we obtain the diagram

$$\begin{array}{ccc} & & H^p(V;\, Z) \\ & & \downarrow{\scriptstyle i^*} \\ H^p(V) & \xrightarrow{\lambda} & H^p(V;\, C) \\ \downarrow{\scriptstyle \Pi} & & \downarrow{\scriptstyle i^*} \\ H^{0,p}(V) & \xrightarrow{\mu} & H^p(V;\, \Omega^0), \end{array}$$

where $H^p(V)$ is the space of harmonic p-forms on V, $H^{0,p}(V)$ is the subspace of harmonic form of type $(0, p)$, λ, μ are isomorphisms onto, and $\mu\Pi = i^*\lambda$. The isomorphism λ is the classical one defined originally by Hodge, and the isomorphism μ is due to Dolbeault.[3] Applying this to the case $p = 2$, we see that, for given $c \in H^2(V;\, Z)$, $(i^*)^2c (\in H^2(V;\, \Omega^0))$ is equal to zero if and only if $\Pi\lambda^{-1}i^*c$ is zero, while, since $\lambda^{-1}i^*c = Hc$, $\Pi\lambda^{-1}i^*c = \Pi_{0,2}Hc$. On the other hand, we infer from (2) that there exists F with $c(F) = c$ if and only if $(i^*)^2c = 0$. Thus we see that there exists F with $c(F) = c$ if and only if $\Pi_{0,2}Hc = 0$. Since c, and hence Hc, is real, $\Pi_{0,2}Hc = 0$ implies $\Pi_{2,0}Hc = 0$. Consequently there exists F with $c(F) = c$ if and only if the harmonic part Hc of c is of type $(1, 1)$, q. e. d.

Thus *the homomorphism* $F \to c(F)$ *maps* $\mathfrak{F}$ *onto the subgroup* $H^2_{(1,1)}(V;\, Z)$ *of* $H^2(V;\, Z)$ *consisting of all* c *such that* $Hc = \Pi_{1,1}Hc$. Letting $\mathfrak{P}$ be the kernel of this homomorphism, we obtain

$$\mathfrak{F}/\mathfrak{P} = H^2_{(1,1)}(V;\, Z) \tag{3}$$

4. Picard Varieties.—Consider a bundle $F \in \mathfrak{P}$ defined by f_{jk}. Then, choosing rational integers c_{jk} suitably, we infer that the holomorphic functions $h_{jk} = \log f_{jk} + 2\pi i c_{jk}$ satisfy

$$h_{jk} + h_{kl} + h_{lj} = 0, \qquad \text{in } U_j \cap U_k \cap U_l.$$

Thus the system $\{h_{jk}\}$ defines an element h_N of $H^1(N; \Omega^0)$ for each covering $\mathfrak{U}$. Moreover, if $\mathfrak{U} < \mathfrak{U}'$, the canonical homomorphism $H^1(N'; \Omega^0) \to H^1(N; \Omega^0)$ maps $h_{N'}$ onto h_N. Thus $h = \lim_N h_N$ is a well-defined element of $H^1(V; \Omega^0)$. The diagram

$$\begin{array}{ccc} & & H^1(V; Z) \\ & & \downarrow i^* \\ H^1(V) & \xrightarrow{\lambda} & H^1(V; C) \\ \Pi \downarrow & & \downarrow i^* \\ H^{0,1}(V) & \xrightarrow{\mu} & H^1(V; \Omega^0) \end{array}$$

shows that $\bar{\alpha} = \mu^{-1}h \in H^{0,1}(V) \subset H^1(V)$ is the conjugate of a simple differential α of the first kind on V and $h = i^*\lambda\bar{\alpha}$. In this manner, each bundle $F \in \mathfrak{B}$ is determined by an element $\bar{\alpha} \in H^{0,1}(V)$ and, moreover, the correspondence $\bar{\alpha} \to F$ is a homomorphism from $H^{0,1}(V)$ onto $\mathfrak{B}$. It is clear that the bundle F is trivial if and only if $h = (i^*)^2(2\pi ic)$, $c \in H^1(V; Z)$, while the relation $h = (i^*)^2(2\pi ic)$ is equivalent to $\bar{\alpha}(= \mu^{-1}h) = 2\pi i\Pi_{0,1}Hc$. Thus we see that the bundle F is trivial if and only if the corresponding $\bar{\alpha}$ has the form $\bar{\alpha} = 2\pi i\Pi_{0,1}Hc$, $c \in H^1(V; Z)$. Let $2g$ be the first Betti number of V. Then $H^{0,1}(V)$ is a complex vector space of dimension g and

$$\mathfrak{Z} = \{\omega \mid \omega = 2\pi i\Pi_{0,1}Hc, \qquad c \in H^1(V; Z)\} \tag{4}$$

is a discrete subgroup of $H^{0,1}(V)$ generated by $2g$ vectors which are linearly independent with respect to real coefficients. Hence the factor group $H^{0,1}(V)/\mathfrak{Z}$ is a complex torus of dimension g and the isomorphism

$$\mathfrak{B} \cong H^{0,1}(V)/\mathfrak{Z} \tag{5}$$

holds.

In order to determine the explicit form of the correspondence $\bar{\alpha} \to F$, take a point $z(j)$ in each $U_j \in \mathfrak{U}$. Then the element $h_N \in H^1(N; \Omega^0)$ approximating $h = i^*\lambda\bar{\alpha}$ is given by the system of constants

$$\tilde{h}_{jk} = \int_{z(j)}^{z(k)} \bar{\alpha}.$$

Hence the bundle F corresponding to $\bar{\alpha}$ is (equivalent to the bundle) defined by the system of constants

$$\tilde{f}_{jk} = \exp \int_{z(j)}^{z(k)} \bar{\alpha}.$$

Let $\bar{\alpha} = \sum_{\nu=1}^{g} t_\nu \bar{\alpha}_\nu$ where α_ν are a base for the simple differentials of the first kind on V. Then the above formula shows that the bundle $F \in \mathfrak{P}$ depends "analytically" on the parameters $t_1, t_2, \ldots, t_g$. Thus it is reasonable to introduce a complex analytic structure on $\mathfrak{P}$ by identifying $\mathfrak{P}$ with the complex torus $H^{0,1}(V)/\mathfrak{J}$. We call $\mathfrak{P} = H^{0,1}(V)/\mathfrak{J}$ the *Picard variety* attached to the Kähler variety V. This is a generalization of the Picard varieties attached to algebraic varieties over the complex number field.[4]

Thus we have proved the following

THEOREM. *The group $\mathfrak{F}$ of all complex line bundles over a Kähler variety V contains the Picard variety $\mathfrak{P} = H^{0,1}(V)/\mathfrak{J}$ attached to V as a subgroup and the factor group $\mathfrak{F}/\mathfrak{P}$ is isomorphic to the subgroup $H^2_{(1,1)}(V; Z)$ of the 2nd cohomology group $H^2(V; Z)$ with integral coefficients consisting of all $c \in H^2(V; Z)$ whose harmonic parts Hc are of type $(1, 1)$.*

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[1] Chern, S., "Characteristic Classes of Hermitian Manifolds," *Ann. Math.*, **47**, 85–121 (1946).

[2] Séminaire H. Cartan, 1951–52.

[3] Dolbeault, P., "Sur la cohomologie des variétés analytiques complexes," *Compt. rend.* (Paris), **236**, 175–177 (1953).

[4] Chow, W. L., "On Picard Varieties," *Am. J. Math.*, **74**, 895–909 (1952); Igusa, J., "On the Picard Varieties Attached to Algebraic Varieties," *Ibid.*, 1–22; Wiel, A., "On Picard Varieties," *Ibid.*, 865–894.

DIVISOR CLASS GROUPS ON ALGEBRAIC VARIETIES

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In a previous paper[1] we have determined the structure of the group of complex line bundles over a compact Kähler variety. In this paper we show that for an algebraic variety this group is isomorphic to the divisor class group, and we obtain, in particular, a new proof of the Lefschetz-Hodge theorem concerning algebraic cycles. Moreover, we prove Igusa's first and second duality theorems. We continue with the notation of the previous paper.

Let V be a non-singular algebraic variety of dimension n imbedded in a projective space. As was shown in Section 2 of the previous paper, every divisor D on V determines the corresponding complex line bundle $\{D\}$ over V which may be identified with the divisor class of D. In fact,

letting $\mathfrak{U} = \{U_j\}$ be a sufficiently fine finite covering of V and $R_j(D) = R_j(z;\ D)$ be local meromorphic functions defining D in U_j, the bundle $\{D\}$ is determined by the system of non-vanishing holomorphic functions $f_{jk}(D) = R_j(D)/R_k(D)$. Clearly $\{D \pm D'\} = \{D\} \pm \{D'\}$ and therefore the divisor class group may be regarded as a subgroup of the group $\mathfrak{F}$ of complex line bundles.

Let $\Omega(F)$ be the faisceau over V of germs of analytic sections of the bundle F. An element of $\Omega(F)$ is therefore a mapping $z \to \zeta_j(z) \times z$ of a small domain $U_j \subset V$ into $p^{-1}(U_j) = C \times U_j \subset F$, where $\zeta_j(z)$ is a holomorphic function of z. In case $F = \{D\}$, the mapping $\zeta_j(z) \times z \to \zeta_j(z)/R_j(z;\ D)$ maps $\Omega(\{D\})$ isomorphically onto the faisceau $\Omega^0(D)$ of germs of meromorphic functions which are multiples of $-D$. Therefore we have the isomorphisms

$$H^q(V;\ \Omega(\{D\})) \cong H^q(V;\ \Omega^0(D)).$$

In particular we have

$$\dim H^0(V;\ \Omega(\{D\})) = \dim |D| + 1. \tag{1}$$

Let S be a general hyperplane section of V and let $\Omega^n(S)$ be the faisceau of germs of meromorphic n-forms which are multiples of $-S$.

LEMMA: *The cohomology group $H^q(V,\ \Omega^n(S))$ vanishes for $q \geqq 1$.*

This can be proved in the following manner. Let Ω^n be the faisceau over V of holomorphic n-forms and Ω_S^{n-1} the faisceau over S of holomorphic $(n-1)$-forms. Then we have the exact sequence[2]

$$0 \longrightarrow \Omega^n \xrightarrow{i} \Omega^n(S) \xrightarrow{P} \Omega_S^{n-1} \longrightarrow 0,$$

where P denotes the Poincaré residue operator. The corresponding exact cohomology sequence is

$$\cdots \longrightarrow H^{q-1}(S, \Omega_S^{n-1}) \xrightarrow{\delta^*} H^q(V, \Omega^n) \longrightarrow H^q(V, \Omega^n(S)) \longrightarrow \cdots \tag{2}$$

Moreover, we have the commutative diagram

$$\begin{array}{ccc} H^{q-1}(S, \Omega_S^{n-1}) & \xrightarrow{\delta^*} & H^q(V, \Omega^n) \\ \downarrow \mu & & \downarrow \mu \\ H^{n-1,\,q-1}(S) & \xrightarrow{H} & H^{n,\,q}(V), \end{array}$$

where μ is the isomorphism due to Dolbeault and where H is the mapping $\varphi \to H\varphi$, $\varphi \in H^{n-1,\,q-1}(S)$, $H\varphi$ being the harmonic part of φ considered as a current of type (n, q) on V. Considering the linear spaces $H^{n-q,\,0}(V)$,

$H^{n-q,0}(S)$ which are dual to $H^{n,q}(V)$, $H^{n-1,q-1}(S)$ in a canonical manner, we infer that the restriction map

$$H^{n-q,0}(V) \xrightarrow{r} H^{n-q,0}(S)$$

in the adjoint of the map H. Now, by virtue of a theorem of Lefschetz, the map r is an isomorphism into or onto according as $q = 1$ or $q \geqq 2$. Consequently H, and hence δ^*, is a mapping onto or an isomorphism onto according as $q = 1$ or $q \geqq 2$. Therefore we infer from the exact cohomology sequence (2) that $H^q(V, \Omega^n(S)) = 0$ for $q \geqq 1$, q. e. d.

Letting K be the canonical divisor on V, we have the isomorphism $\Omega^0(D) \cong \Omega^n(D - K)$. Combining this with the above lemma, we obtain the following result:[3] *If the complete linear system $|D|$ is sufficiently ample in the sense that $|S| = |D - K|$ is a system of hyperplane sections of V in one of its ambient spaces, then the cohomology groups $H^q(V, \Omega^0(D))$ vanish for $q \geqq 1$.*

THEOREM 1. *For every complex line bundle F over an algebraic variety V, there exists a divisor D on V such that $\{D\} = F$.*

Proof: We prove the theorem by induction on the dimension n of the variety V. First we consider the general case in which $n \geqq 2$. Let S be a general hyperplane section of V which is fixed and let S_m be a general hypersurface section of V of order m. We denote by $F \cdot S$ the restriction of F to S and by $\Omega(F \cdot S)$ the faisceau on S of germs of analytic sections of $F \cdot S$. We have the exact sequence

$$0 \longrightarrow \Omega(F - \{S\}) \xrightarrow{i} \Omega(F) \xrightarrow{r} \Omega(F \cdot S) \xrightarrow{\ast} 0 \quad (3)$$

where i is the inclusion map defined by

$$\zeta_j(z) \times z \longrightarrow R_j(z; S)\, \zeta_j(z) \times z,$$

and where r is the restriction.

Now, let F be a given bundle. Setting

$$F_m = F + \{S_m\} = F_{m-1} + \{S\},$$

we have, by (3), the exact sequence

$$0 \longrightarrow \Omega(F_{m-1}) \xrightarrow{i} \Omega(F_m) \xrightarrow{r} \Omega(F_m \cdot S) \longrightarrow 0$$

and therefore the corresponding exact cohomology sequence

$$\begin{aligned} 0 \longrightarrow H^0(V, \Omega(F_{m-1})) &\xrightarrow{i^*} H^0(V, \Omega(F_m)) \xrightarrow{r^*} H^0(S, \Omega(F_m \cdot S)) \\ &\xrightarrow{\delta^*} H^1(V, \Omega(F_{m-1})) \xrightarrow{i^*} H^1(V, \Omega(F_m)) \xrightarrow{r^*} \\ &\qquad H^1(S, \Omega(F_m \cdot S)) \longrightarrow \cdots \quad (4) \end{aligned}$$

By the inductive hypothesis, there exists a divisor $\bar{D}$ on S such that $\{\bar{D}\} = F \cdot S$ on S. We have therefore

$$F_m \cdot S = F \cdot S + \{S_m\}S = \{\bar{D} + S_m \cdot S\}, \qquad \text{on } S,$$

where $S_m \cdot S$ is the hypersurface section of S cut out by S_m. Obviously the complete linear system $|\bar{D} + S_m \cdot S|$ is sufficiently ample if m is large. Hence, by the above result, there exists an integer m_0 such that

$$H^1(S;\ \Omega(F_m S)) = H^1(S, \Omega^0(\bar{D} + S_m \cdot S)) = 0, \qquad \text{for } m \geqq m_0.$$

Since the cohomology groups $H^q(V, \Omega(F_m))$ have finite dimensions,[4] we therefore obtain from (4)

$$\dim H^0(S, \Omega(F_m \cdot S)) = \dim H^0(V, \Omega(F_m)) - \dim H^0(V, \Omega(F_{m-1})) + \dim H^1(V, \Omega(F_{m-1})) - \dim H^1(V, \Omega(F_m)), \ (m > m_0)$$

Adding both sides of this formula on m, we get

$$\dim H^0(V, \Omega(F_m)) \geqq \sum_{m = m_0 + 1}^{m} \dim H^0(S, \Omega(F_m \cdot S)) - d_0, \qquad (5)$$

where $d_0 = \dim H^1(V, \Omega(F_{m_0}))$. Now, since $F_m \cdot S = \{\bar{D} + S_m \cdot S\}$, we have, by (1),

$$\dim H^0(S, \Omega(F_m \cdot S)) = \dim |\bar{D} + S_m \cdot S| + 1 \to +\infty \text{ for } m \to +\infty.$$

We therefore infer from (5) that

$$\dim H^0(V, \Omega(F_m)) \to +\infty \text{ for } m \to +\infty.$$

This shows that, for large m, the bundle F_m has a global analytic section $\zeta_j(z) \times z$ different from $0 \times z$. Letting D_m be the divisor on V defined by $\zeta_j(z) = 0$ in each U_j, we infer readily that $F_m = \{D_m\}$. Hence, setting $D = D_m - S_m$, we obtain $F = \{D\}$.

In the case $n = 1$, $H^0(S, \Omega(F_m \cdot S)) = \Omega(F_m \cdot S) = \Omega(S)$ is a linear space of dimension $s \geqq 1$, s being the degree of the divisor S, and $H^1(S, \Omega(F_m \cdot S))$ vanishes for any m. Hence, applying the above argument to this case, we infer the existence of D with $\{D\} = F$. This completes our inductive proof.

Letting $H_{2n-2}(V; Z)$ be the $(2n-2)$th homology group of V with integral coefficients Z, we have the canonical isomorphism $H^2(V; Z) \cong H_{2n-2}(V; Z)$ which maps each cohomology class $c \in H^2(V; Z)$ onto its dual $*c \in H_{2n-2}(V; Z)$. Since $H(*c) = Hc$, the mapping $c \to *c$ gives the isomorphism

$$H^2_{(1,1)}(V; Z) \cong H^{(1,1)}_{2n-2}(V; Z), \qquad (6)$$

where $H^{(1,1)}_{2n-2}(V; Z)$ is the subgroup of $H_{2n-2}(V; Z)$ consisting of all homology classes whose harmonic parts are of type (1, 1). Each divisor

D is an integral $(2n-2)$-cycle on V. Moreover, it can be shown that the homology class of D is equal to the dual of the characteristic class of the bundle $\{D\}$:

$$D \in *c(\{D\}). \tag{7}$$

In fact, we choose a suitable covering $\mathfrak{U} = \{U_j\}$ and a suitable corresponding simplicial decomposition of V in which each vertex $z(j)$ lies in U_j and we denote by $s(jkl)$ the simplex with the vertices $z(j)$, $z(k)$, $z(l)$ where $U_j \cap U_k \cap U_l$ is not empty. Let e_j be the dual $2n$-cell of $z(j)$ and let D be defined by $R_j(z; D)$. Then we have

$$2\pi i I(D, s(jkl)) = \Delta_j \log R_j(z; D) + \Delta_k \log R_k(z; D) + \Delta_l \log R_l(z; D) = \log f_{jk}(D) + \log f_{kl}(D) + \log f_{lj}(D) = 2\pi i c_{jkl},$$

where $\Delta_j \log R_j(z; D)$ is the variation of $\log R_j(z; D)$ around the boundary of $e_j \cap s(jkl)$ and where the branches of $\log f_{jk}(D)$, ... are chosen in the obvious manner. This proves the formula (7) above. Formula (7) shows that every divisor D belongs to the group $H^{(1,1)}_{2n-2}(V; Z)$.

Now, let $\mathfrak{G}$ be the additive group of all divisors D on V, $\mathfrak{G}_a$ the subgroup of $\mathfrak{G}$ consisting of all D which are homologous to zero with respect to integral coefficients, and let $\mathfrak{G}_l$ be the subgroup of $\mathfrak{G}_a$ of all D which are linearly equivalent to zero. Then it follows from Theorem 1 that

$$\mathfrak{G}/\mathfrak{G}_l = \mathfrak{F} \tag{8}$$

In view of (7), D belongs to $\mathfrak{G}_a$ if and only if $\{D\} \in \mathfrak{P}$. Hence we get

THEOREM 2. *(Igusa's first duality theorem.)*[5] *We have*

$$\mathfrak{G}_a/\mathfrak{G}_l = \mathfrak{P} \tag{9}$$

where $\mathfrak{P} = H^{0,1}(V)/\mathfrak{Z}$ *is the Picard variety attached to* V. Combining (6), (8), and (9) with the theorem in the previous paper, we get $\mathfrak{G}/\mathfrak{G}_a \cong H^{(1,1)}_{2n-2}(V; Z)$, while (7) shows that this isomorphism coincides with the natural inclusion map $\mathfrak{G}/\mathfrak{G}_a \xrightarrow{i} H^{(1,1)}_{2n-2}(V; Z)$. Hence we obtain the following

THEOREM 3. *The factor group* $\mathfrak{G}/\mathfrak{G}_a$ *coincides with* $H^{(1,1)}_{2n-2}(V; Z)$:

$$\mathfrak{G}/\mathfrak{G}_a = H^{(1,1)}_{2n-2}(V; Z) \tag{10}$$

This leads immediately to the following criterion for algebraic $(2n-2)$-cycles due to Lefschetz and Hodge:[6]

THEOREM 4. *An integral* $(2n-2)$*-cycle* Γ *on an algebraic variety* V *of dimension* n *is homologous to a divisor if and only if the harmonic part* $H\Gamma$ *of* Γ *is of type* $(1, 1)$.

Let $\mathfrak{G}_r$ be the subgroup of $\mathfrak{G}$ consisting of all divisors which are homologous to zero with respect to rational coefficients, and let $T_q(V)$ be the qth torsion group of V. Since $T_{2n-2}(V) \subseteq H^{(1,1)}_{2n-2}(V; Z)$, we infer from

(10) that $\mathfrak{S}_r/\mathfrak{S}_a = T_{2n-2}(V)$, while $T_{2n-2}(V)$ is dual to $T_1(V)$. Consequently we obtain the following

THEOREM 5. *(Igusa's second duality theorem.)*[7] *The factor group* $\mathfrak{S}_r/\mathfrak{S}_a$ *is dual to the first torsion group* $T_1(V)$ *of* V.

[1] Kodaira, K., and Spencer, D. C., these PROCEEDINGS, **39**, 868–872 (1953).

[2] Kodaira, K., and Spencer, D. C., "On Arithmetic Genera of Algebraic Varieties," *Ibid.*, **39**, 641–649 (1953).

[3] This result has been pointed out by J. P. Serre as a consequence of his duality theorem.

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THE GROWTH OF AMBLYSTOMA CHIMERAS

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Communicated by V. C. Twitty, June 1, 1953

The production of chimeras by combining anterior and posterior halves of *Amblystoma tigrinum* and *A. punctatum* embryos at the tail bud stages was shown to be possible by Stone in 1934.[1] Although normally *A. tigrinum* far surpasses *A. punctatum* in growth rate and size, Stone reported that the anterior component of the chimeras regulated the growth of the combination, at least up to metamorphosis. According to his findings, the "anterior half was always species dominant in its effect." That is, the anterior punctatum halves reduced, and the anterior tigrinum halves increased, the intrinsic growth rate of the posterior halves. Although not specifically stated in Stone's report of his work, it seemed to be implied that this species dominance of the anterior half resulted in the formation of harmoniously proportioned chimeras.

These results, as Twitty later pointed out in his review of size-controlling factors,[2] were in contrast with the results obtained in heteroplastic grafts of eyes and limbs between the two species. In these two instances the transplanted organs maintained their normal growth rates and achieved normal size.[3, 4] There are, however, several instances in which size effects are exerted between structures intimately related in their development, such as the development of harmoniously proportioned eyes when the optic vesicle and lens epidermis are interchanged between *A. tigrinum* and *A. puncta-*

tum.[5] Since many organ systems were involved in the growth of Stone's chimeras, Twitty suggested that the total regulation might represent the cumulative effect of several contributing factors. But he pointed out that before a final interpretation could be given Stone's results, further experimentation was needed.

Accordingly, the present writer has repeated and extended Stone's experiments employing *A. tigrinum* and *A. punctatum* embryos both from Tennessee and from the Chicago area. Because it seemed conceivable that the dominance of the anterior part might vary with the proportions in which the two components were united, the fusions were made at levels ranging from in front of the gills to behind the anus. Most of the animals and controls were fed maximally, but some cases were purposely not.

The results differ significantly from those achieved by Stone. Except for slight regulation at the line of fusion, necessary for the connection of the two unequally proportioned parts, in no instance has the anterior component been observed to exert a significant effect on the size of the posterior part. (See figures.) Each part has consistently maintained up to metamorphosis a size an hereditary pattern characteristic of its species irrespective of the level at which the union was made. Although some evidence has been accumulated to show that partial size adjustment in the posterior part does occur six to eight months after metamorphosis, the findings up to metamorphosis are in agreement with those obtained from heteroplastic grafts of eyes and limbs between these two species, and lend themselves to similar analytical study and interpretation.

Results of further anatomical and histological studies will be forthcoming.

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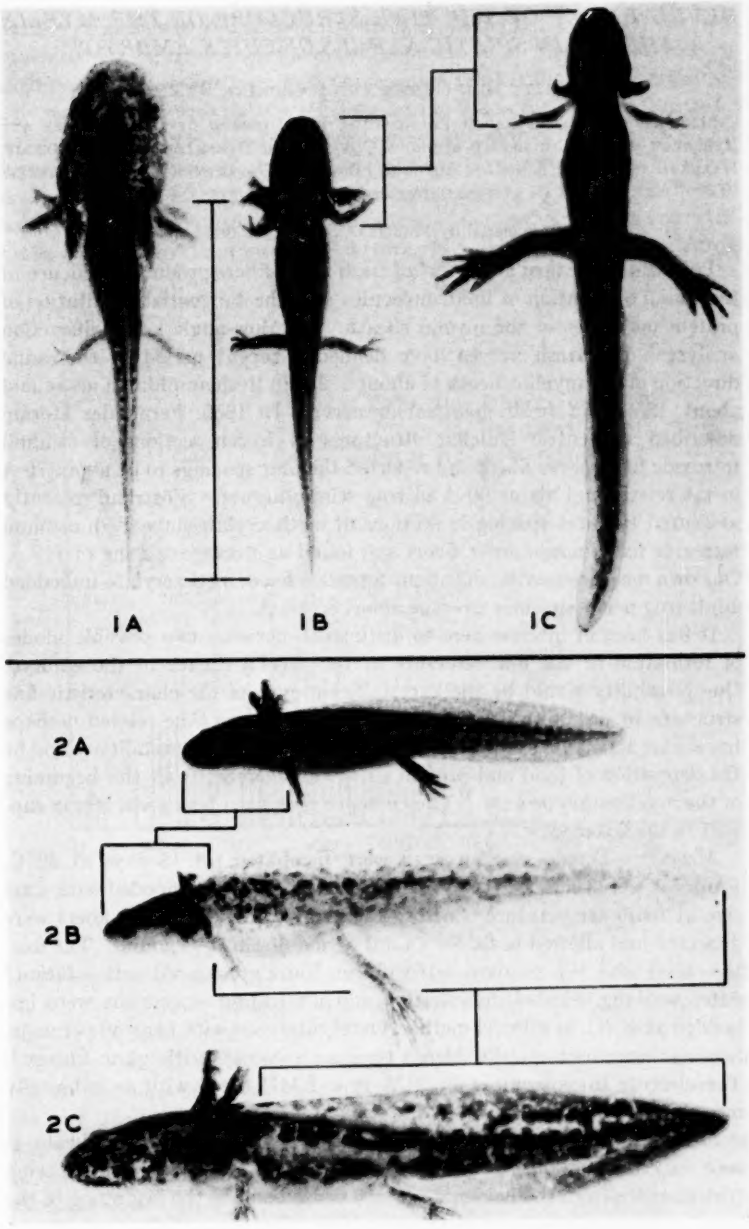
FIGURE 1

(*A*) *A. tigrinum* head combined with *A. punctatum* body. (*B*) *A. punctatum* control. (*C*) *A. punctatum* anterior part combined with *A. tigrinum* posterior part.

FIGURE 2

(*A*) *A. punctatum* control. (*B*) Lateral view of chimera shown in figure 1 (*C*). (*C*) *A. tigrinum* control. Brackets will assist the reader in comparing the size of the chimeric and control components.


1A
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2C

DEVELOPMENT OF THE FINE STRUCTURE OF THE MYELIN SHEATH IN SCIATIC NERVES OF CHICK EMBRYOS*

BY BETTY BEN GEREN AND JOSEPHINE RASKIND

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Communicated by Francis O. Schmitt, May 25, 1953

Polarization optical studies[1, 2] of fresh nerve fibers produced evidence of the radial orientation of lipid molecules and the tangential orientation of protein molecules in the myelin sheath. The low-angle x-ray diffraction analyses[3, 4] of fresh nerves have defined a repeat period in the radial direction of the myelin sheath of about 172 A in fresh amphibian nerve and about 185 A in fresh mammalian nerve. In 1950, Fernández-Morán[5] described concentric lamellar structures in frozen sections of osmium tetroxide fixed nerve fibers and reported the unit spacings to be about 70 A in rat sciatic and about 60 A in frog sciatic nerves. Sjöstrand[6] recently measured the unit spacing in sections of methacrylate-imbedded, osmium tetroxide fixed mouse nerve fibers and found an average spacing of 119 A. Our own measurements[7] of osmium-tetroxide fixed, methacrylate-imbedded adult frog nerve spacings average about 85–95 A.

It has been of interest here to distinguish between two possible modes of formation of the fine structure of the myelin sheath in the embryo. One possibility would be the "crystallizing out" of the characteristic fine structure in accumulated lipid and protein at some time related perhaps to cellular activity or axonal function. An alternative possibility would be the deposition of lipid and protein as oriented layers from the beginning of the myelination process. The evidence presented here gives strong support to the latter view.

Methods.—Fertile chicken eggs were incubated for 18 days at 39°C. Embryos were removed, the sciatic nerves exposed and flooded with fixative at room temperature. After preliminary blackening, the fibers were dissected and allowed to fix for a total period of 1 to 1½ hours. The fixative used was 1% osmium tetroxide in Hank's balanced salt solution.[8] After washing, alcohol dehydration and infiltration, specimens were imbedded at 45°C. in *n*-butyl methacrylate[9] catalyzed with benzoyl peroxide. Sections were cut on the Minot-type microtome[10] with glass knives.[11] The electron microscope is an RCA type EMU fitted with an externally movable objective aperture.[12]

Results.—The concentric lamellar structure of the myelin sheath is seen in cross section of the embryonic nerve fibers. The unit spacing (the distance from the beginning of one dense band to the beginning of the

next) was measured in undisrupted areas of 22 fibers (1056 total spacings measured). Figure 1 shows a distribution curve of these measurements, each position representing the average measurement for 3 to 14 spacings. The mean is 97 A with an average deviation of 9 A. Figure 2 (*a*) illustrates a region in a cross-section of a sciatic nerve, and figures 2 (*b*) and 2 (*c*) are higher magnifications of indicated areas in the myelin sheath. The thickness of the dense lines in the spacing varies according to focus, thickness of section, orientation of the fiber in the plane of section, and degree of astigmatism caused by the objective aperture. In our best in-focus pictures

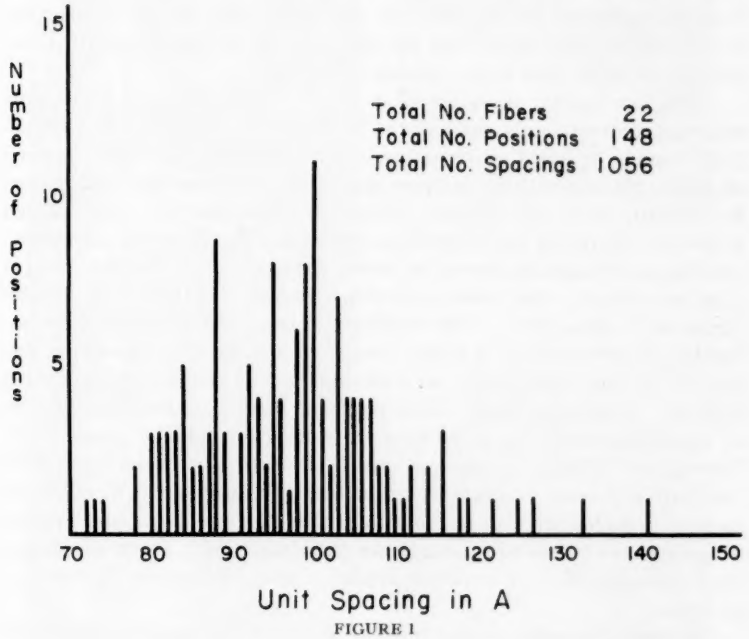

FIGURE 1

of through-focus series the width of this dense line varies between 20 and 50 A. No regularly arranged structures were observed in the less dense band of the unit repeat.

Within a single sciatic nerve are fibers with varying numbers of layers in the myelin sheath. Preliminary study of earlier ages of development (14 and 15 days) reveals many fibers with 2 to 12 concentric lamellae whereas in the 18-day material described here most fibers have 24 or more lamellae, although a few are seen with lesser numbers of layers. It is thus apparent that the increase in myelination of nerve fibers in the embryo

proceeds by the addition, in an oriented manner, of lipids and proteins to the surface of the axonal projection within the cytoplasm of the Schwann cell. The Schwann cells in the early (14-day) embryonic nerve trunk are extremely large compared to the size of the individual myelinating fibers which appear as large Schwann cell cytoplasmic inclusions. This is in marked contrast to the satellite type of appearance of the Schwann cell around the adult fiber. Detailed studies of the earlier (14- and 15-day) stages will be reported later.

Discussion.—It is apparent that the myelin sheath has well-ordered fine structure in the 18-day chick embryo. Our observations indicate that from the beginning, the deposition of lipid and protein about the axon occurs in well-ordered layers and the thickness of the myelin sheath is increased by an increase in the number of layers.

The unit spacing measures 97 ± 9 A. One might expect that a unit structure approximately one-half the large period shown by x-ray diffraction[3, 4] would be based on repeating lipid double layers arranged in some alternating fashion with the predicted tangential protein lamellae, as suggested by Schmitt, Bear, and Palmer. However, several factors make detailed studies of the period and correlation with the x-ray data still premature. Swelling and shrinking may occur during fixation with undefined changes of water content. One observes obvious areas of distortion of the myelin sheath as in figure 2 (*c*). The swelling and gross distortion of tissues imbedded in methacrylate is easily recognized and such specimens are discarded, but the evaluation of small changes in volume due to imbedding is difficult. Fernández-Morán's data on frozen sections of fixed material are of significance here, but more data are needed for detailed comparisons. Very slight distortion of the specimen from heating in the electron beam can further prevent accurate measurements. Measurements of fibers cut somewhat diagonally with respect to the fiber axis would give greater than actual values for the unit spacing in the radial direction. In our specimens, the myelin sheath and its spacings appear to be compressed in the direction of cutting.

Our lack of knowledge regarding the nature of the reactions of osmium tetroxide with tissue components further limits detailed analysis of the tissue fine structure as revealed by the present methods.

Correlation of the fine structure of developing nerve fibers with the time of appearance of conduction cannot be attempted yet, but further advances in techniques and in interpretation of results may make such correlation possible. Investigations of this type may also permit analyses of factors influencing myelin formation at levels of resolution that allow interpretation of the lipid and protein molecular arrangement.

Conclusions.—The forming myelin sheath of embryonic nerve fiber has a repeat spacing similar to that of the myelin sheath of mature fibers.

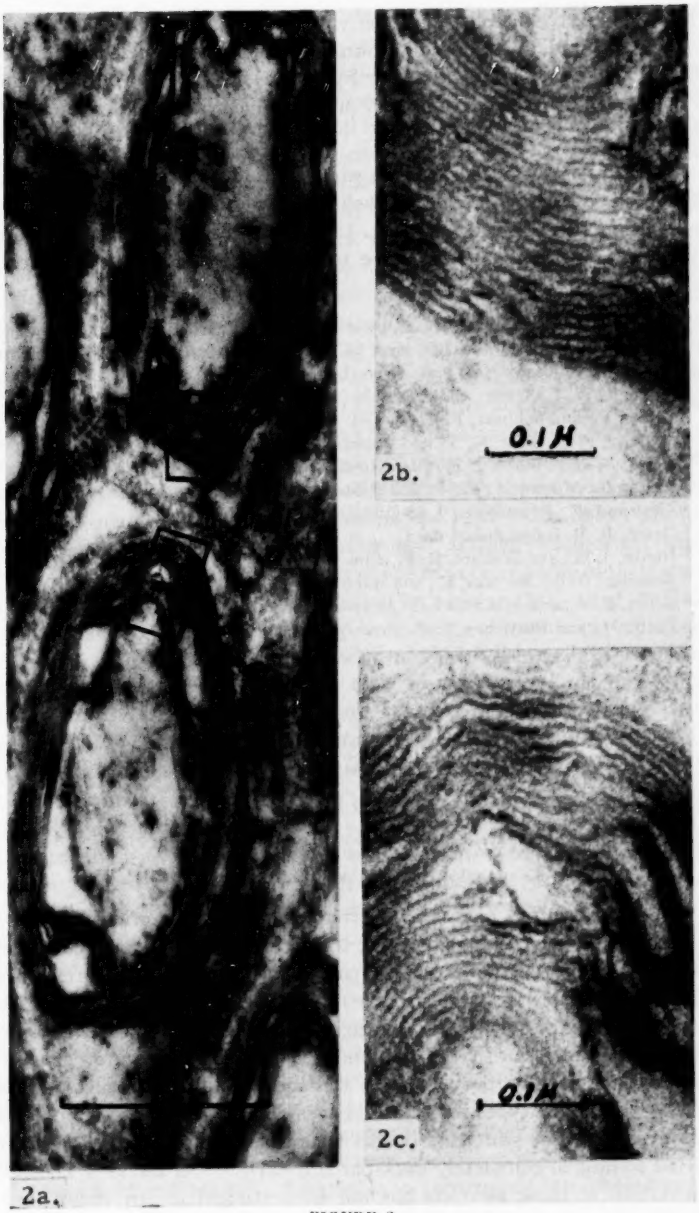

FIGURE 2

The forming myelin consists of concentric lamellar structures arranged as a sheath around the axon as it courses in the cytoplasm of the Schwann cell. The thickness of the forming myelin sheath is dependent on the number of concentric lamellae and the younger the fiber the smaller the number of unit layers.

Acknowledgments.—The authors would like to thank Drs. Marie Jakus, Cecil Hall, and Hugh Huxley for helpful discussions of technical problems and Miss Phyllis Hoffman and Mr. Donald Misch for technical assistance. The encouragement and guidance of Prof. F. O. Schmitt is gratefully acknowledged.

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ACOUSTIC ORIENTATION IN THE OIL BIRD, STEATORNIS

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Communicated by H. A. Bethe, June 15, 1953

The discovery that bats use high frequency sounds to orient themselves during flight has naturally given rise to speculation and inquiry concerning the possible existence of similar types of orientation in nocturnal birds. The limited studies of owls that have been made to date indicate that these birds rely primarily on vision, although their dark-adapted thresholds may be slightly lower than that of the human eye.[1] There are a few species of birds that habitually roost in caves, and some build their nests well inside caverns of various types where the light is dim at best. For example, the "edible bird's nest swiftlets" (genus Collocalia) of southeast Asia are reported to nest in completely dark parts of caves.[2] As far as I know the orientation of these swiftlets has not been studied in any detail, but I

hope that the observations described below may serve to stimulate such investigations in the future.

In northern South America there is another bird which roosts during the day almost exculsively in caves, and flies out to feed only during the night. This bird, *Steatornis caripensis*, the oil bird or guacharo, was described by von Humboldt in 1800, and it attracted wide interest among zoologists during the 19th century because it has no close relatives and is consequently placed in a family by itself. The bird was given its name because of the high fat content of the nestlings which were gathered in large numbers to yield edible fat. Steatornis is somewhat similar in structure to the family Caprimulgidae (nighthawks, whip-poor-wills, goatsuckers, and the like), but has a hawk-like bill and several anatomical pecularities such as the most extreme form of the paired, bronchial syrinx to be found in birds.[3]

Over the past several years I have attempted to collect all available information about the roosting places of these birds in caves, in particular the degree of darkness prevailing where they roost and fly. Out of several eye-witness accounts only a few stated with any assurance that the birds penetrate beyond the twilight zone, and it is certain that many of the nests, probably a majority, are located where some light is available. A few reports, however, gave strong indication that at least some of the birds fly into regions of total darkness. One of the most convincing of these came from William H. Phelps, Jr., on the basis of his visits to the Cave of the Guacharos at Caripe, State of Monagas, Venezuela—the same cavern where von Humboldt first discovered the birds in 1800. As a result of our correspondence Mr. Phelps arranged for E. de Bellard in 1951 to expose some film for 4 or 5 minutes in the innermost chamber of the cave that is occupied by the oil birds. This roll of film appeared after development to be virtually undarkened, although a second, unexposed strip was very slightly more transparent.

A still more convincing report was later received from O. P. Pearson in a letter describing a visit to las Cuevas de Tulini, Province of Tolima, Colombia, July 27–28, 1950. The following pertinent passage may well be quoted: "The ceiling (of the cave) is in places 75 or more feet high and there are additional holes and cracks containing many bats.... At dusk and after, two oil birds flew for perhaps a half hour near the ceiling of the cave...sometimes being chased by large bats (probably *Phyllostomus discolor*).... These birds made a loud snapping sound as well as a raucous rattling cry.... I looked through the cave at some time in the middle of the night.... The oil birds were flying in the cave before dawn—even with my eyes dark adapted I could not see my hand in front of my face. The birds were making a snapping sound as they flew, like a wooden ratchet, 5 to 10 clacks per second, perhaps an echolocation mechanism."

On March 14 and 15, 1953, I was able to accompany Mr. Phelps to the

Cave of the Guacharos at Caripe and to study this problem directly. Some of the apparatus used to study the high frequency sounds of bats was taken to the cave, and a portable, high fidelity tape recorder was also used to record the sounds made by the oil birds while flying through the cave. The other members of the party, all of whom rendered assistance in these studies were: Mrs. W. H. Phelps, Jr., Dr. and Mrs. W. McCrudy, Dr. G. Zuloaga and his son, G. Zuloaga, Jr. The trip was an extension of the author's studies of acoustic orientation in tropical bats supported in the Canal Zone by the Office of Naval Research. But all expenses of the trip from Panama to Caripe were generously contributed by Mr. Phelps, and without his support these studies would not have been possible.

Our first step on arrival at the cave was to walk into the deepest chamber where the oil birds roost and nest (650 meters from the entrance) and to remain there without lights of any sort for 25 minutes. This period was ample to attain complete dark adaptation, especially since it followed 15 or 20 minutes during which we walked into the cave with only the light of flashlights and gasoline lanterns. Even after this complete dark adaptation none of us could detect any signs of light. On the following day I repeated this observation both during the morning and afternoon, and in addition exposed several frames of Eastman Super XX film for varying periods of time by removing the lens mount of a camera and pointing the emulsion toward the entrance to this chamber of the cave. Frames exposed for 5 and 9 minutes (the longest exposures made) showed no detectable darkening when developed. Adjacent frames had not been exposed at all, and even though they underwent the same development procedure as the test frames, no line of demarcation could be seen in the finished roll between exposed and control areas. This chamber is 75 to 100 ft. high, and the birds roost on ledges near the ceiling. But the passage leading into this room is lower, and at the position where we dark-adapted ourselves, and where the film was exposed, there was more opportunity for light to penetrate than could possibly have reached the upper space where the birds were flying. It thus seems clear that these birds habitually fly in total darkness where vision cannot play any role in their orientation.

Our next step was to make tape recordings of the sounds emitted by the birds in this innermost occupied chamber while we remained as quiet as possible without lights on the floor of the cave 50 to 75 ft. below them. The equipment used for this purpose was a Western Electric 640AA condenser microphone, a cathode follower, a Ballantine model 220 decade amplifier, and an Amplifier Corporation of America model 610E tape recorder, all battery operated. The protective grid was used over the microphone, but no horn was employed. The frequency response of this system was in all probability uniform from 50 to 15,000 c. p. s. within ± 6 db.

All eye-witness accounts of oil bird colonies in caves, beginning with von

Humboldt's[4] have stressed the clamor made by the birds when disturbed by visitors to the cave. Even to one thus forewarned the noise was hair-raising, so that one could feel only sympathy for Humboldt's Indian guides who refused to advance beyond a certain point in the cave because they believed the oil birds were in fact ghosts of their ancestors and that the inner parts of the cavern could only be entered by the dead. The sounds that we heard and recorded were of several sorts. Some were obviously call notes from birds perched on the ledges, others were squawks and screeches made as the birds flew back and forth above us, and still others consisted of the rapidly repeated "clucks" or "clicks" which Pearson had evidently heard in Colombia. The latter were especially noticeable when a bird flew down closer to where we stood.

Of special interest were the sounds made by the oil birds as they spontaneously flew out through the cave in the evening. To record them the microphone was held at the top of a large rock 50–60 ft. below the ceiling of the cave and within 100 yd. of the entrance. In addition to the apparatus used in the inner room, the sounds picked up by the condenser microphone were also studied by means of a DuMont model 304H cathode-ray oscillograph, powered by storage battery and vibrator. The oscillograph screen was photographed with a Bell-Howell Model 70DA 16-mm. motion picture camera in order to obtain graphic records such as figure 1. For some records and tape recordings a battery-operated Spencer-Kennedy model 301 variable electronic filter was used between the amplifier and oscillograph; but unfiltered records and tape recordings were also obtained under all conditions of interest. These unfiltered records showed no appreciable components below 2000 c. p. s. and consequently the filter was used at 2000 c. p. s. high pass to improve the signal-to-noise ratio in records such as figures 1 and 2.

The most striking fact about the sounds we heard and recorded as the birds flew out from the cave was the almost complete absence of calls and screeches such as those that predominated in the roosting chambers during the day. Instead there were almost nothing but loud, sharp clicks, repeated rapidly and almost continuously as the birds flew past. Only at long intervals would one of the hundreds of birds emit a longer duration sound like those that had bombarded our ears in the inner chamber.

A typical oscillograph record of these clicks is shown in figure 1; this was photographed directly from the oscillograph screen at the cave with the 2000 c. p. s. high pass filter. This picture is typical of hundreds of frames of the 16-mm. film, and while the wave-form varies to some extent from one record to another, the duration, frequency, and interval between pulses are approximately constant.

As will be explained below, such records as figure 1 show that most of the energy in the clicks lay well within the frequency range of the tape

recorder. Hence the tape recordings provide a continuous record of adequate fidelity for most purposes. Figure 2 is a cathode-ray oscillograph record made from one of these tapes using a camera which moved the film continuously and which had a shutter that remained open throughout the period of film exposure. Study of many similar records, and watching the oscillograph while the tapes were being played back, show that figure 2 is typical of the sounds recorded on the tape when only one bird was within range of the microphone.

The frequency of these clicks emitted by the oil birds lies in most cases between 6000 and 10,000 c. p. s. No accurate time standard was available for the studies at the cave, so that the time scale for the direct cathode-ray oscillograph records such as figure 1 is probably less accurate than those like figure 2 made from the tapes. The average of 33 frequency measurements in records similar to figure 2 is 7300 c. p. s., the extremes of this series being 6100 and 8750. In pulses of such short duration the very concept of frequency begins to lose some of its usual meaning, and many of the records suggest that even within a single pulse the waves may differ slightly in their period or wave-length. Harmonics can be noted in the direct oscillograph records such as figure 1, and their presence interferes with precise measurements of frequency. The true amplitude of the harmonics is somewhat greater than the photographs indicate, because the sensitivity of the 640AA microphone declines at approximately 12 db per octave at frequencies above 12 kc.[5] But even when allowance is made for this factor it is still clear that the principal sound energy is contained in the fundamental waves that lie well within the range of human hearing. While we have no direct knowledge of the frequency range of hearing in Steatornis, birds in general are insensitive to frequencies above 20,000 c. p. s.[6]

It should be emphasized in this connection that the apparatus which yielded figure 1 is the same as that which I have previously used for studies of the high frequency sounds of bats.[7] Hence any strong components above 20 kc. would certainly be visible in the records, provided they reached the microphone. To be sure, the distance of about 50 ft. from bird to microphone would have caused considerable attenuation of ultrasonic frequencies; for instance, at 50,000 c. p. s. the attenuation in the air would be approximately 30 db.[5] It would thus be possible to contend that during flight out through the cave the birds emitted sounds having strong components at ultrasonic frequencies, but that these components were so greatly attenuated by 50 ft. of air that they are not apparent in the records such as figure 1. But oscillograph records of the clicks emitted by one of the freshly caught birds to be described below revealed no signs of any such high frequency components, at least none having amplitudes approaching those of the fundamental frequencies below 10,000 c. p. s. Yet the sounds emitted by the only bird studied at close range by means of the os-

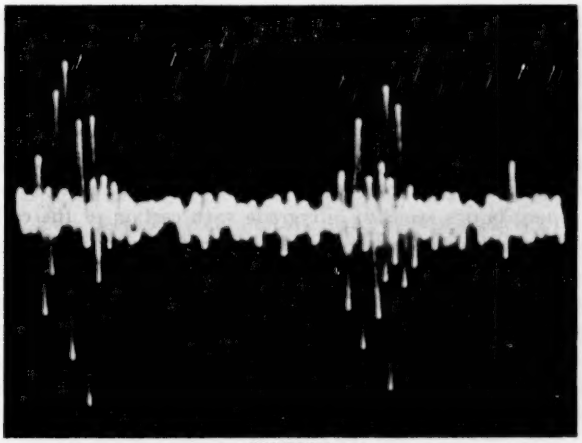

FIGURE 1

Cathode-ray oscillograph record of two clicks emitted by an oil bird as it flew out through the entrance of the cave. Each sweep occupied 5 msec. (0.005 sec.). The camera shutter was open for 16 msec. The frequency response of the apparatus was nearly uniform from 2000 to 10,000 c. p. s. and declined above that frequency at approximately 12 db per octave.

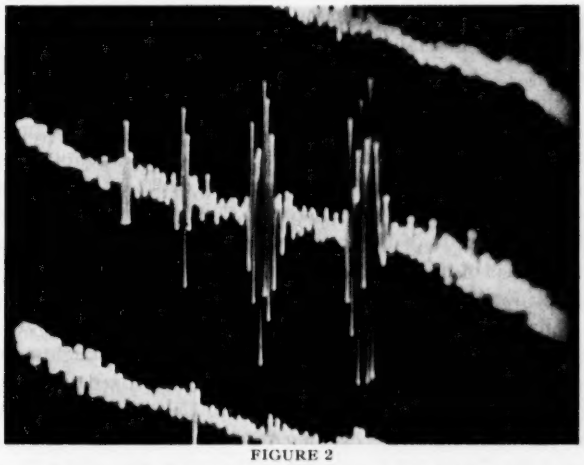

FIGURE 2

Oscillograph record made from tape recording. In this picture the film moved vertically while the sweep circuit moved the oscillograph trace from left to right, each sweep requiring 16.7 msec. The frequency response of the apparatus used was approximately uniform from 2000 to 15,000 c.p. s.

cillograph were probably only a limited part of its vocal repertoire, and further studies would be necessary before one could completely exclude the possibility that high frequency components are important under some conditions.

The clicks emitted by the oil birds do not occur at a regular rate, but rather come in short bursts such as the one illustrated in figure 2. The number of clicks in such bursts varied from as few as two up to six or more; and the longer bursts seem to intergrade with certain of the call notes, although their true relation to other sounds made by the birds cannot be determined without further study. Within the bursts, however, the interval between clicks did not vary widely. Out of 43 intervals measured the average is 2.6 milliseconds, the extremes of this series being 1.7 and 4.4 msec.

The amplitude or sound pressure of these sounds could not be measured since the apparatus was not accurately calibrated and because the distance from bird to microphone and the angle of incidence were beyond our control. But the clicks were readily audible even when the birds were 200 yd. away; and all of our records were obtained as they flew at least 50 ft. above the microphone. The duration of the individual clicks is approximately 1 msec. Occasionally there are very short pulses containing only two or three waves (such as the first pulse of figure 2); but the longest pulse discovered among hundreds examined to date had a duration of almost exactly 1.5 msec.

It thus seemed clear that the oil birds fly through the virtually complete darkness of caves, and that while doing so they emit pulses of sound very similar to those used by many species of bats, but having a lower frequency—well within the range of human hearing and that of most birds. This evidence alone is not conclusive, although it certainly supports the idea that these birds guide their flight through dark caves by the type of acoustic orientation I have called echolocation.[8] We therefore carried out a few simple experiments of the same type as those which had established the auditory basis of obstacle avoidance in bats.[9]

For this purpose Mr. Phelps caught four live oil birds by means of a trammel net. Observations in the cave indicated that any significant experiments required a dark room, for in the light of our flashlights the birds greatly reduced the number of clicks and sometimes stopped them altogether. It therefore seemed best to carry the birds to Caripito where a darkroom was improvised the following evening through the cooperation of Dr. G. Zuloaga and the Creole Oil Company. This room was about 10 or 12 ft. square and 8 ft. high; and the birds flew about inside it reasonably well despite the fact that they had been in captivity for 24 hours and had not eaten any appreciable amount of food since the night before their capture. Either in light or in darkness they avoided any direct collisions with the

walls, although they did brush their wing tips from time to time as they turned in the room, which was only about four times their wingspread in its dimensions. In the dark their movements could easily be followed by the noise of their wingbeats as well as by the clicks which sounded approximately like those emitted while flying out through the cave. None of the birds was able to avoid frequent collisions with an electric light cord suspended from the ceiling; but it is quite possible that an object of this size could have been detected and avoided by oil birds flying under more natural conditions.

The following tests were carried out with each of the three strongest birds, one at a time. After being allowed to fly about the room two or three times (in the dark) to show that it was able to avoid the walls successfully, each bird was captured and its ears plugged with absorbent cotton and Duco cement. The cotton was packed fairly tightly into the internal ear canal but only as far as the first bend. The cement was then applied in a double layer over the cotton and the immediately adjoining skin. The bird was held quietly for two or three minutes to allow partial drying of the cement and then released in the dark. After two to five such flights the plugs were removed and the bird's flight again observed in the dark with the ear canals unencumbered.

In every case the bird with its ears plugged flew directly against the first wall it came to, and noisy collisions were the immediate result of all attempts to fly. Yet all three birds recovered their previous skill once the plugs had been removed. The general weakness of the birds made it impossible to carry out more than a few flight tests in each condition—intact, ears plugged, and intact again after removal of the plugs—but the results were uniform and consistent. The strongest bird gave the most impressive performance; for it continued to fly vigorously even after the ears were stopped, and began to show signs of weakness only after several collisions with the walls. Yet it recovered its skill and vigor after removal of the plugs, and at the end of the observations seemed as agile as the other birds had been at the beginning of their tests.

With the room light turned on the untreated birds flew skilfully, but emitted many fewer clicks than when flying in the dark. One bird was flown in the light with its ears plugged. It emitted very few clicks, and had no difficulty avoiding the walls of the room. Evidently when light is available the oil birds orient themselves by vision, as one might expect from the fact that their eyes are roughly as large as those of other birds of similar size. When flying in the dark with ears plugged, however, all three birds clicked vigorously, and almost without interruption.

While this evidence should ideally be supplemented by more thorough studies of orientation under a wider variety of conditions, it already seems clear that sound plays an essential role in guiding the flight of Steatornis

through the dark. Not only can this bird now be added to the list of animals known to orient themselves by echolocation, but the sound frequencies employed by Steatornis are far lower than those used by any of the bats yet studied. Yet the oil bird, like most bats, employs *pulsed* sounds having a duration so short that the whole wave train in a single click extends over less than a meter as the sound travels through the air. Short pulses have the advantage that echoes return even from objects at close range during a silent period when they can more easily be heard. While Möhres[10] (1953) has recently described a special type of echolocation in bats of the family Rhinolophidae (the horseshoe bats of the Old World), in which pulses lasting roughly one-tenth second are utilized, all other bats yet investigated use pulses lasting only a few milliseconds.

The fact that the oil bird can orient itself successfully during flight through dark caves by echolocation based on sounds having a frequency of roughly 7000 c. p. s. reinforces the idea that similar types of orientation are quite possible in our own species. Blind men are known to make extensive use of sound in their orientation, and recent experiments show that hearing is the most important sense used by the blind to detect objects at a distance.[11] The fact that bats can guide their flight by echolocation based on ultrasonic sounds has perhaps seemed to have only an academic interest for blind people. Perhaps the success of the oil bird will prove more encouraging. I even venture to hope it may stimulate efforts to improve the methods of acoustic orientation now used by the blind. One important aspect of such attempts should surely be a study of various types of sound that might be emitted in order to produce useful echoes, and a promising first step would be the use of very short pulses, or clicks, like those of bats and oil birds.

Summary.—1. The oil bird, *Steatornis caripensis* Humboldt roosts during the daytime in caves, and many of the birds choose nesting sites in total darkness where vision could not be used for their orientation. This was established for the innermost occupied chambers of the Cave of the Guacharos at Caripe, Venezuela, by (1) noting that even after 25 minutes of dark adaptation no light could be seen as we stood under the birds' nesting ledges, and (2) by exposure film for 9 minutes without causing any detectable darkening of the emulsion.

2. When flying about inside the cave the oil birds make a variety of sounds, but when flying out for the night the predominant type is a sharp click, repeated in short bursts while the bird is on the wing. When light is available the clicks are much less frequent or cease altogether.

3. The individual click consists of a very few sound waves having a frequency of about 7000 c. p. s. (average of 33 measurements 7300, extremes 6100 and 8750 c. p. s.). The duration of each click is about 1 msec. the shortest measured to date being 0.3 msec., and the longest 1.5

msec. The interval between clicks within the short bursts varied between 1.7 and 4.4 msec. in a series of 43 measurements, the average being 2.6 msec. While no measurements of intensity were feasible, the clicks were loud enough to be easily audible at a distance of 200 yd. within the cave. Except for their lower frequency these pulses of sound are very similar to those used by most genera of insectivorous bats for their acoustic orientation.

4. The external ear canals of three captive oil birds were plugged with absorbent cotton covered with Duco cement, and all three birds became disoriented when flying in the dark with their ears plugged. Before, and immediately after this treatment, they flew about within a small dark room avoiding collisions with the walls. But when their ears were plugged they never turned away from a wall and collided with every object encountered.

5. It thus seems clear that Steatornis guides its flight through dark caves by a type of acoustic orientation similar to that used by bats, but the oil bird employs for this purpose short pulses of sound that lie well within the frequency range of human hearing.

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